

What happened to social science?

Paradoxically the social sciences remain under a cloud when governments and their electors are more worried by social problems than for many years. Should not the social sciences exert themselves?

ONCE upon a time, just a decade or so ago as it happens, there was a small but quickly-growing army of men and women persuaded that it should be possible to help make the world a better place not simply by making it more wealthy but by enabling it to understand its objectives and its motives. For one reason or another, the once-growing army has been shrinking for several years, and has also fallen into silence when vociferousness used to be its habit. Those concerned were called social scientists, people who at the academic level were differently recognizable as economists and psychoanalysts and who, otherwise, worked at tasks as different as management consultancy and probation officers. The curious circumstance is not so much that they have fallen so silent but that they have done so when the need for people with their pretensions is, if anything, greater than ever. Why should this be?

No doubt there are special circumstances. In Britain, for example, the citadel of the social sciences used to be the Social Science Research Council, rechristened against its will in 1983 as the Economic and Social Research Council by Sir Keith Joseph (now Lord Joseph); it became unpopular with its sponsors for a variety of reasons, some of them political. (To be fair, the council never really had the resources to be a citadel; it was a turret at best.)

Perhaps the most serious charge that can be laid against social scientists with ambitions in research is that it is even harder in their arid fields than in the natural sciences to demonstrate a link between what they spend on their projects and the welfare of the nation whose taxpayers happen to be supporting them. Part of the trouble is that even when social science research is oriented towards the improvement of public policy, government officials have both a vested interest and the right to overlook the implied advice — and then to complain that their rejection shows the projects to have been ill-conceived. But it is not over-malign to guess that the more serious damage has been done by changed demeanour towards the introspections of the social scientists effected in the past few years in most Western democracies, reflected in the matter-of-fact impatience of the governments they elect.

Paradox

The consequence is a curious paradox: governments are more than ever alarmed about newly emerging social problems, but are inclined to deal with them empirically, by trial and (often) error. In the United States, for example, the abuse of drugs is on everybody's mind, and may yet be an election issue, but the government is inclined to turn towards the military, not the social scientists. Some people are worried sick by the quality of public education, but the preferred solutions tend to be organized around the qualifications of teachers and their salaries. Elsewhere (but also in the United States), the spread of AIDS is at the head of most social agendas, but easier access to a source of condoms is the most common nostrum. In Britain, where soccer hooliganism is threatening to turn what is called the "national sport" (at which the nation concerned is not especially successful) into a television spectacle only, the remedy seems to

be that hooligans (and others) should be issued with identity cards. Alcohol abuse, belatedly acknowledged as the social burden it is, is as likely in the prevailing mood to be dealt with punitively.

The obvious risk in the widespread adoption of these mechanistic solutions for accurately perceived social problems is that, as with all remedies that are directed at the removal of symptoms rather than of causes, they may not be solutions at all. Requiring those who attend football matches in Britain to be equipped with identity cards will probably do more to put struggling or marginally successful football teams out of business than to get rid of hooliganism, for which there are many other outlets still not exploited. Would it not be better, in the circumstances, at least to attempt to understand why these tribal rituals persist and even flourish in prosperous societies, and to look for ways of diverting the energy they consume into more constructive activities? Would it not be useful, and perhaps even beneficial, to know more about the reasons why people choose to take drugs? Or to drink too much? In short, is this not a time when the apparently vanished social scientists should be riding high?

Unfashionable though it may be, that view deserves more attention than it receives in the present climate. The practitioners and their academic colleagues will be quick to blame governments for indifference and insensitivity, and there is much force in that complaint. But it is also fair to say that the practitioners themselves, but especially their academic colleagues, have been slothful in seeking to demonstrate their potential usefulness at this time of unprecedented prosperity and uncertainty for most of the societies in which they used to be entrenched. Governments are fond of blaming the natural sciences for having failed productive industry, but are not in a mood to blame social scientists for failing society. But others will if they do not exert themselves. □

Cambridge independent?

Trinity College's generosity towards its university should offer hope to other British universities.

THE striking feature of the decision by Trinity College, Cambridge, to set up a trust fund for the benefit of the university of which it is an autonomous part is not the amount of money (see page 93) but the mechanism involved. The objective is to provide a university largely dependent on public funds with an independent income, however small by the yardstick of its annual need. Many at Cambridge (and elsewhere) will now be busily calculating just how much more would be needed to secure complete independence. The answers will be depressing — on the assumption that present rules still apply. But what if the government were to change the rules, letting students carry with them the cost of their tuition? That would neatly get the universities off the British government's back and the government off the back of the universities. Should not the government give that a try? □



Heated response to US drought

- No signs of break in drought crisis
- Greenhouse effect enters political arena

Washington

A LITTLE rain fell on portions of the United States last week, but according to the latest forecast from the National Weather Service Climate Analysis Center, the hot dry weather that has been blanketing the country will continue throughout this week, exacerbating the drought conditions plaguing the west, northern plains, midwest and south-east states.

Although the drought is not directly related to the build-up of greenhouse gases which some claim has caused average global temperatures to rise, the juxtaposition of the two events may become more tightly coupled politically than they are scientifically.

Officials at the US Department of Agriculture (USDA) noted warning signs of the possibility of a drought as early as last February. Norton Strommen, chief meteorologist at USDA, says the low level of the snowpack and water supply were signs that, without favourable weather, severe crop damage would result.

When the jetstream failed to move south from Canada in June, hot dry weather prevailed through the critical growing period. Crops such as corn (maize), which is extremely sensitive to heat during anthesis, are being especially hard hit. Half of the Illinois corn crop has already been ruined, and more will be lost if there is no rain.

Ironically, the same warning signs from the snowpack and water level were there in the winter of 1987, but timely spring rains brought record crop yields last year. It is also true that, if soil moisture had been normal last winter, the hot dry conditions would not have had the same disastrous consequences.

There are clear signs that the drought will get worse before it gets better. According to data from the US Geological Survey, some 60 per cent of 181 index stations around the country report stream flow well below normal for June. By comparison, only 38 per cent of stations were reporting below normal flow in May. Approximately three dozen stations are reporting the lowest water levels on record.

In its latest drought advisory notice issued on 6 July, the Climate Analysis Center long-term forecast predicted that

the Palmer Drought Index — a measure of drought conditions — would continue to be at minus 3 for the northern plains, west and mid-west, indicating severe to extreme drought conditions.

Despite a spate of record temperatures, Strommen says the current conditions are not out of line with previous hot summers. He says that although greenhouse warming may be occurring, there is no doubt that the current weather patterns could have occurred without any increased atmospheric CO₂.

Strommen believes that increased forest harvesting, coupled with the spread of "civilization" bringing an increase in paved surfaces is a more likely culprit. If solar radiation is not absorbed by evapotranspiration, he says, it will turn into sensible heat.

James Firor, a climatologist at the National Center for Atmospheric Research in Boulder, Colorado, agrees that

average temperatures are on the way.

Even if this year's weather conditions are not directly related to greenhouse warming, they may become inextricably linked in public perception. Congress has been holding hearings on this question, and there are daily news reports that catalogue the scope of the problem and attempt to explain its antecedents.

Stephen Schneider, also of NCAR, notes that while bureaucrats move cautiously, insisting on cost-benefit analyses before taking steps to correct a problem, politicians act in response to their reading of public perceptions. As the drought remains in the news, the public belief that it is related to the buildup of greenhouse gasses may grow. By September, as forest fires result from the dry conditions and the presidential campaign moves into full swing, climate control could well become a campaign issue.

The government has meanwhile convened the Presidential Drought Task Force to coordinate the federal response to the crisis. Norman Rosenberg, director of the Climate Resources Program at



NOAA's satellite photographs from 1986 and 1988 show the extent of the retreat of vegetation.

it is not possible to say with certainty that any specific weather pattern is related to the greenhouse effect. But he does believe there is evidence for greater frequency of temperature extremes, such as the one now occurring.

Firor also points to evidence from time series analysis of global climate models that a rise in the average global temperature of 0.5 degrees centigrade will result in increased fluctuations skewed towards warmer temperatures, not a smooth, gradual warming. He says he is well aware that next year, the pattern could change dramatically, with an unusually cool wet summer. "We suspect that even during the ice age there were hot years", he says, but that does not change the fact that warmer

Resources for the Future, says that poor responses to droughts in the 1970s convinced federal officials of the need for better organization, but it is not clear that there is yet the concentrated effort he believes is needed.

Rosenberg's programme is involved in designing strategies that will make US agriculture less vulnerable to extreme weather conditions. Wider use of winter wheat, development of hardier plant species, renewed use of wind breaks, and improved irrigation technology are all strategies that could mitigate damage from future droughts. But Rosenberg warns that the impetus for change often evaporates when rain relieves a drought.

Joseph Palca

Soviet basic science wins friends but also some enemies

London

It has now emerged that Dr Gurii Marchuk, president of the Soviet Academy of Sciences, pleaded for more support for basic research in an address to the Special Conference of the Communist Party of the USSR last month. Marchuk's speech has just been published in full in *Pravda*.

Echoing Mr Mikhail Gorbachev's keynote speech, Marchuk declared that basic research, "the basis of all science and of all scientific and technical progress", had long been neglected (and inadequately supported) in the Soviet Union. The consequence is that Soviet science is "not entirely" ready to shoulder the tasks called for by *perestroika*.

In a frank comparison between Soviet science and that of the United States, Marchuk told the conference that, while the numbers employed in research may be roughly equal, the "capital-to-labour" ratio in the United States is at least three times as great as in the Soviet Union, with the result that US science is more productive.

Marchuk insisted that state funds will have to be the chief source of support for basic research, dismissing in the process the claims of those who have been urging "cooperative measures" for the support of science. But he also followed Mr Gorbachev's argument that science will be effective only if it is rid of "petty tutelage" and interference.

He urged that holders of all major posts in the scientific establishment (including himself) should be elected and hold office for no more than 10 years. To promote *glasnost* in science, he is also planning to put to the Soviet government in the near future a plan for a weekly science newspaper.

Dr Evgenii Chazov, all-union minister of health and another speaker at the special conference, complained of the misdirection of funds during the Brezhnev "era of stagnation", complaining of the frequent boasts that the Soviet Union had more physicians and hospitals than any other country although it came fiftieth in the world in the infant mortality league, behind Mauritius and Barbados and thirty-second in the life-expectancy tables.

Chazov also told the conference the story of the town of Tynda, on the new Baikal-Amur mainline railway whose local authority built a railway station in marble and aluminium to impress visiting delegations, but left the regional hospital in a series of army huts.

The rector of Moscow University, Dr Anatolii Logunov, pleaded for more money: "We are absolute paupers in

modern equipment", he said, and went on to complain that even the university's attempt to buy up-to-date computers of Soviet manufacture had enmeshed it in negotiations with the all-union ministry of finance.

Logunov also complained that the "Lysenko virus" is still alive, and that "certain people try to silence others who think differently". That may have been a heartfelt remark; Logunov is himself the author of a theory of relativity that commands few friends. Logunov asked that the universities should have "their hands untied" by dispensing with the State Committee for Public Education, formed only last autumn. He urged a broad programme of international cooperation to allow hundreds of thousands of Soviet students to study abroad, not just ones and twos as at present.

Not all those who addressed the special conference were as trustful. The director of the Ivanovo Machine Building Production Association, Mr Kabaidze, reproached Academician Marchuk for not demanding that science should be self-financing. He complained that Soviet industry had to support too many research institutes which, with a few exceptions, were failing to produce results, and said that a vast state research sector was not necessarily the best way to produce the high technology on which the future depends. "I don't think I've ever heard of the South Korean Research Institute", he said, "but South Korea is now in the top ten of the industrialized nations".

The newly established State Committee for the Protection of the Environment complained through its president, Dr F.T. Morgun, that scientists themselves are responsible for many of the Soviet Union's environmental problems. Borys Oleynek, chairman of the Ukrainian Writers' Union, was fearful for what would happen when the Danube-Dnieper Canal would discharge "all of Europe's effluent" into the Ukraine's chief river, and also complained that public anxiety about nuclear power stations had not been taken seriously enough.

Even if the balance of the science presentations to the special conference may have been in favour of autonomy and local decision-making, not all the talk went that way. Thus Academician Primakov, from the Soviet Academy's Institute of the World Economy, attacked the notion that each of the Soviet republics should run its own research programme, regardless of resources and personnel. Rather, Primakov said, Soviet science should be "an integrating force" in Soviet life.

Vera Rich

Trust in Trinity

TRINITY College, Cambridge is to set up a trust fund to help Cambridge University out of its financial difficulties. The 28 Cambridge colleges, some of which have substantial fortunes, have till now kept their finances separate from the university, which is funded by the government. Now Trinity is setting a precedent by stepping in to help the university which has suffered under cutbacks in government funding. The trust, to be called the Isaac Newton Trust, will be complementary to the university's own trust and will set apart several million pounds to benefit education and research in the university. Trinity hopes this will set an example which other colleges may follow.

C.McG.

Tired of waiting

THE "secrecy barrier" on Jewish scientists wishing to emigrate from the Soviet Union may be reduced to five years, according to participants in a seminar of the International Bar Association in Moscow. Rumours of such a change have been current in Moscow for some time, and Soviet officials told the lawyers that there was "some truth" in them. But implementing the changes, the officials said, will be a long process, as hard-line bureaucrats, opposed to Mikhail Gorbachev's reforms, are trying to block it — it may take longer than five years to introduce the "five-year upper limit".

Jewish *refusnik* scientists in Moscow are sceptical about the alleged changes. Although the number of scientists allowed to emigrate has increased considerably in the past few months, the *refusniks* point out that these were predominantly from the backlog of cases where the scientist concerned had been so long out of professional employment (in some cases as long as 15 years) that to claim that he or she still possessed "secret" information was simply ludicrous. People whose cases are regarded by the authorities as more "complicated" are now being told that they cannot expect a decision before 1992. Moreover, the concept of the 'brain drain', normally applied to the developing nations, is still being invoked by some Soviet officials.

Inquiry completed

THE planned inquiry into the conduct of the experiments reported in *Nature* two weeks ago by Dr Jacques Benveniste was conducted in Paris last week. Those who visited his laboratory were Mr James Randi, the professional magician who is also a Macarthur Fellow, his assistant Mr José Alvarez, the editor of *Nature*, Mr John Maddox, and Mr Walter W. Stewart from the Institute of Allergy and Infectious Diseases of the US National Institutes of Health. In consultation with Dr Benveniste, a report will be published in *Nature* on 28 July.

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British physicians brood on HIV testing and 'designer children'

London

PHYSICIANS in Britain agreed last week that patients should be tested for the human immunodeficiency virus (HIV) only for clinical reasons and with their consent. According to this decision, physicians testing for the virus without the patient's consent must believe it to be in the patient's best interest, and be prepared to defend their actions in a court of law and before the General Medical Council.

In passing this resolution at its annual conference last week, the British Medical Association (BMA) reversed last year's decision to allow testing for HIV without necessarily seeking patients' consent. Lawyers advised the BMA that this could lead to legal action against doctors.

At the same time, last week's conference resolved to promote anonymous screening for HIV in order to gather more information about the true extent of infection in Britain. Otherwise, physicians fear that the public will become increasingly immune to warnings about AIDS. But they agreed that screening would have to be done in such a way that tests could not be traced to an individual; the BMA criticized a screening programme of pregnant women at St Bartholomew's Hospital, London, on the grounds that it is small enough for individuals to be identified.

The conference also decided that, in order to slow down the spread of the virus, condoms should be made freely available in prisons, despite the legal dilemma this presents for the government: homosexual acts are legal only in private and prisons do not offer privacy.

The conference was also concerned at the amount of personal information physicians are being required to give to insurance companies. Dr John Dawson, head of the BMA's professional division, said that more companies were insisting on tests for HIV before agreeing to mortgages of more than £50,000. The conference resolved that patients should see the questions that doctors are to be asked on a medical report and sign their consent.

The BMA conference also sanctioned guidelines to physicians on the implications of genetic engineering. Dr Ian Jessiman spoke of the possible dangers of allowing developments in genetics to proceed without regulation. While genetic substitution could bring substantial benefits, he said, it could also have drawbacks, including the production of what he called 'designer children' with pre-selected characteristics. He also warned that as testing for genetic defects became more sophisticated, people might demand abortion for increasingly trivial reasons. And he warned of the ecological implications of the accidental release of dangerous viruses into the environment. Dr Dawson added that it was important to consider now what the future implications of interfering with the human gene pool might be.

The BMA is also to investigate the effects of pesticides on human health. The conference agreed that more information is necessary on the effects of long-term exposure to low levels of pesticides, on which the investigation will concentrate.

Christine McGourty

Renewed efforts by French haemophiliacs on compensation

Paris

THE death from AIDS last week of the president of the French Hemophiliac Association, André Leroux, aged 49, adds further poignancy to the association's unsuccessful demands for compensation for haemophiliacs contaminated with the human immunodeficiency virus (HIV) following treatment with blood products. France has so far not decided what its position should be.

Since 1985, France has screened all blood offered for transfusion for the presence of antibodies to HIV. But current health ministry estimates show that at least 226 people were infected following transfusions before these measures were introduced. In addition, an estimated 1,500 (almost half) of the country's haemophiliacs have been infected by con-

taminated blood products and 50 have already died from AIDS.

Before the recent change of government, the former health minister, Michèle Barzach, defended the position that the ministry is not legally bound to compensate individuals infected through contaminated blood or blood products. As such treatments are not classified as drugs, and so no charge of negligence can be filed, the question of liability remains unresolved. In the meantime, Barzach had promised to give FF300,000 (£28,600) to the French Hemophiliac Association to help members suffering from AIDS, but this money has so far not been released.

In France, when the national haemophiliac association initially approached government, few seropositive members

Bavarian AIDS ruling will go to appeal

Munich

A CONTROVERSIAL ruling in a bizarre court case seems to be a new departure in the West German legal system's approach to AIDS. To the dismay of Bavarian hardliners, the decision of a district court (*Amtsgericht*) in the city of Kempten on 1 July may make it more difficult to prosecute AIDS virus carriers who purposefully transmit the virus.

The case involves a 29-year-old Italian HIV carrier, who was accused of "attempting to inflict dangerous bodily harm" by having unprotected sexual intercourse (without using a condom) with his 16-year-old German girlfriend. The girl was aware of his condition and was not infected, although the couple had relations over a six-month period in 1987.

The court ruled that even if the girl had been infected with HIV, this could not be construed as "harmful" in the absence of an outbreak of AIDS. The court also declared that its ruling should not be allowed to set a precedent, stating that each case needs to be judged on its own merits. The Kempten public prosecutor, Walter Hofmaier, was dissatisfied with both the narrow scope of the ruling as well as the court's tacit approval of the couple's behaviour, and says he will appeal. The ruling, if it stands, might remove the legal basis for the prosecution of AIDS "desperadoes" who purposely try to infect others.

Hofmaier also says that the state is required to protect human life, and could not accept such careless and self-endangering behaviour on the part of an HIV carrier or his or her sexual partner. Suicide, however, is legal in West Germany.

A consensus is forming in Bavaria that a new law is needed to deal specifically with AIDS and virus carriers. So far, the government has prosecuted cases such as this one using laws regarding assault, attempted murder or the Federal Epidemic Law, as occurred in another recent case (see *Nature* 333, 585; 1988). Steven Dickman

were willing to take legal action. Following the death of its president, the association intends to redouble its efforts. But, as other countries have found, the question of eligibility for compensation is complex. Should awards be restricted to seropositive haemophiliacs, or extended to their wives and families? Should the size of awards reflect the victim's life expectancy? Should non-haemophiliacs infected by transfusion also be compensated?

Meanwhile the French blood transfusion society wants to encourage donors to give plasma, rather than blood. Plasma does not carry the same risks of infection and has a wider range of pharmaceutical applications.

Peter Cole

Soviets' pair of Mars probes paving the way for man

London & Washington

THE Soviet Union's most ambitious planetary mission ever got off to a successful start last Thursday with the launch of Phobos-1, the first of two 6-tonne spacecraft that will rendezvous next year with Mars. The launch of Phobos-2 was scheduled for Tuesday this week.

When they reach Mars, the spacecraft will survey the planet from orbit for 120 days, taking photographs, recording infrared and gamma-ray spectrograms, and collecting data on the solar wind and solar radiation.

Phobos-2 will then transfer to an orbit about the martian moon Phobos and carry out similar observations. Finally, next May it will make a 20-minute flyby of the satellite, approaching to within 50 metres of the surface, moving with a relative velocity of 2 to 5 metres a second — almost walking pace.

During the flyby Phobos-2 will use a laser to ionize soil on Phobos, while a mass spectrometer will analyse the resulting vapour. A radar beam will also take soundings of the moon for surface structure analysis.

After completing the survey, Phobos-2 will drop a "long-life autonomous station" to the surface of Phobos. This has been assigned a 3-month programme using X-ray and alpha backscatter spectrometry to study surface composition. Cameras will take close-up pictures and a seismometer will measure seismic activity.

Phobos-2 will also drop a mobile lander to the surface of Phobos. Dubbed the hopper, this lander will make 20-metre leaps on spring-loaded legs, giving a wider distribution of sample sites.

The United States and Soviet Union have been recently making friendly overtures towards each other over Mars. Each country will have scientific teams participating in the other's Mars missions. The next US mission is the Mars Observer, scheduled for launch in 1992.

The National Aeronautics and Space Administration (NASA) is not providing any hardware for the Phobos mission, but the US space agency does have a significant part to play in helping the Phobos spacecraft arrive at the right place at the right time.

Using very-long baseline interferometry (VLBI), NASA's deep space network will pinpoint the location of the Phobos spacecraft starting this autumn. This technique, now standard for tracking spacecraft, uses two of the three deep space network antennas (one in southern California, one in Spain, and one in Canberra, Australia) to record signals from the spacecraft simultaneously to

determine its angular position relative to a reference frame provided by quasar radio sources.

In April, communications hardware from Phobos was tested at the Goldstone (California) tracking site to assure that the US and Soviet equipment was compatible. NASA will continue to use the radio beacon on the Phobos landers to conduct VLBI studies, but there are two other instruments on the landers that NASA scientists will have access to. One will be used to study Phobos' wobble — libration — during its 7 hour and 39 minute-rotational period. The rotational period is the same as its orbital period, so one face is constantly pointed towards Mars.

But slight vibrations that will be detected by measuring the angular position of the Sun from Mars will be used to deduce the internal distribution of mass of Phobos. This should help determine whether Phobos is a captured asteroid.

A second instrument will be used for ranging studies. A relayed signal transmitted from Earth will allow precise determination of Phobos' distance to an accuracy of 10 metres.

Robert Preston of JPL, who serves as NASA's chief scientist for Phobos activities, says Phobos is rapidly — in celestial terms — spiralling in towards Mars, and in another 50 million years may crash into the planet or break apart under tidal forces. Precise knowledge of Phobos's orbit should also give a better idea of the Martian gravitational field, and may provide information about the mass of nearby asteroids.

An even more exciting experiment, according to Preston, will be a more precise determination of the gravitational constant. Preston says the the Solar System provides a marvellous laboratory for conducting experiments on general relativity. It may even be possible to detect changes in the gravitational constant by comparing Phobos data with similar data collected by the US Viking landers in the 1970s.

Soviet commentators are placing great emphasis on two aspects of the flight. One is the extensive international participation. Nineteen nations are involved, either as suppliers of experiments and hardware or as trackers including the United States, the United Kingdom, Australia, Sweden, Finland, Ireland, West Germany and Yugoslavia, as well as the Comecon block. The other is the idea that this is another step towards a manned mission to Mars. During the past three years, record-breaking stays aboard the *Salyut* and *Mir* orbital stations have been officially described as the necessary first step to Mars.

Joseph Palca & Vera Rich

NASA criticized on safety

Washington

WITH the space shuttle Discovery on the pad, ready for launching in September, NASA's commitment to the maintenance of a strong safety management and risk assessment programme is being questioned. In a report published last week, an *ad hoc* committee chaired by Charles Mertz of NASA headquarters and Joyce McDevitt, a private consultant, warned against a lapse into the attitudes of "business as usual" once Discovery is safely in orbit.

The committee, asked by NASA to judge how well it has dealt with criticism of its previous attention to safety matters, says that for the moment internal discussion of risks and their remedies is unfettered and that morale is high. It found no fault in plans for Discovery's impending launch. But the committee is plainly unsure that the present commitment to making safety the overriding con-



The space shuttle Columbia, on the move last week into the Orbital Processing Facility vacated by Discovery. The date for Discovery's next launch has now been put back to the first week in September. Meanwhile, Columbia — seen above with some parts missing and with plastic covers to protect it from the elements — will be refurbished with the latest safety improvements.

cern in the resumption of shuttle flights will continue past the first re-launch.

Acknowledging that in the two and a half years since the Challenger disaster, many senior managers have been devoted full-time to safety issues, and that the procedures for risk control have been completely rewritten, the report warns that when the need to keep to a regular schedule reasserts itself, people will return to their usual duties and there will be little time to review and alter procedures.

To guard against laxity, the committee argues that more permanent staff specializing in safety questions are needed, and that personnel training and assessment should formally cover safety management. Because the driving force behind recent efforts has been largely individual enthusiasm motivated by public concern, the report says there is a danger that if institutional changes are not made now, the present commitment to safety could evaporate.

David Lindley

Göttingen physics: can an historic department be saved?

Göttingen

THE eminent faculty of physics at the University of Göttingen, built up in the early 1900s through clever appointments of the Prussian ministry of culture and grants from the Rockefeller Foundation, is being threatened by outside forces. The problems, brought on in part by the critical economic situation in the *Land* (state) of Lower Saxony, are symptomatic of the difficulties facing many universities in northern West Germany.

Physicists at the University of Göttingen spoke last week of the danger of a *Götterdämmerung* — 'twilight of the gods' — that could drive their faculty, and perhaps the entire university, into irreversible decline.

Lower Saxony is suffering particularly from setbacks in agriculture and oil, two of its largest economic resources. Oil prices have dropped; and a court decision a few years ago required Lower Saxony's government to share its oil profits with the other *Länder*. Similar difficulties exist in neighbouring North Rhine-Westphalia (see *Nature* 332, 298; 1988).

Universities all over West Germany have been filled far beyond capacity since the early 1980s and the number of students has unexpectedly continued to rise in 1988. Göttingen currently has 30,000 students, compared with 3,000 in the 1930s.

The situation in northern Germany contrasts with that in Bavaria and Baden-Württemberg, which inherited an abundance of technology-oriented industry (such as Siemens in Munich) in the realignment of companies in West Germany after the Second World War. These southern *Länder*, which used to be

thought of as agricultural backwaters, are now booming.

Members of the physics faculty in Göttingen say that it has not completely recovered from the effects of the Nazi era. Due to the banning of Jews from the civil service in 1933, Göttingen lost Max Born, James Franck, Richard Courant and many other theoretical physicists and mathematicians in one blow.

The current austerity measures include a cut in the number of tenured faculty and a reduction of at least 3.5 per cent in expenditures for shorter-term personnel over four years. These cuts brought students and professors onto the streets in May 1987.

In the view of Göttingen physicist Klaus-Peter Lieb, Göttingen faces a critical period in the next few years. A wave of retirements will mean that the university will face tough choices in hiring replacements and paying their not insignificant equipment costs.

Lieb admitted that the university is well provided with both outside funding and the infrastructure required to do good research. But continuing budget cuts might start an irreversible slide into mediocrity. Lieb and the other physicists are clamouring for the recognition of the special status of Göttingen — Lower Saxony's oldest and largest university — from the government.

The Lower Saxony Science Ministry has established a 'University Structure Commission' of experts to investigate the options for restructuring the educational system. Its report, expected by the end of the year, may encourage the government to reconsider its priorities.

Steven Dickman

University cuts in the 1990s

London

THE financial squeeze on British universities in the 1980s has prompted university heads to lay down new guidelines for pricing university contracts. Under the new system, research in universities will be more expensive for companies and government departments, which hitherto have paid at rates well below market value and well below the real cost of the contracts to the universities.

A review by the Committee of Vice-Chancellors and Principals (CVCP) points out that, when universities are taking on more outside contracts in a bid to increase their income, undercharging means that more contracts lead to increased deficits.

Universities are advised by the University Grants Committee (UGC) to price outside contracts by calculating the direct cost and adding 40 per cent to cover overheads. But in reality, most universities charge much less than this, allowing only about 12 per cent on average for overheads according to the CVCP.

One reason that universities undercharge is their fear of losing contracts; another is that, in academic circles, charging for overheads is considered a luxury. With the total annual income from universities' contracts at between £150 and £200 million this luxury costs them around about £40 million a year.

The CVCP suggests that charging about 60 per cent of the direct cost of a contract would cover the overheads on recurrent costs, such as salaries. If universities were also to charge overheads on capital costs, such as buildings, then the total would be around 105 per cent, though these figures would vary between departments and between universities. The new system could bring universities almost £200 million more each year.

CVCP stresses that these proposals are flexible. If a university feels a contract is of long-term benefit, it should be free to charge less. And, conversely, if the university wants to make a profit, it should charge more. The point the committee emphasizes is that universities must be aware of whether or not they are subsidizing industry. The CVCP say that, at present, 95 per cent of them are doing so unawares.

The proposals follow an interim CVCP report, last year, to which industry generally responded favourably. But industries also said they would expect a more professional service from universities.

Later this month, this year's CVCP report will go to representatives of industry and other bodies as well as to finance officers of universities.

Christine McGourty

Australian fraud inquiry under way

Sydney

A FORMER chief justice of Australia will chair a special committee convened to investigate allegations of scientific fraud against Dr William McBride, founder and honorary director of the private research institute Foundation 41. The committee will also include two medical academics.

Foundation 41 has convened the investigation itself after several other relevant organizations declined to do so (see *Nature* 332, 671; 1988). The research in question involves a study of hyoscine, an anticholinergic medication, and its potential for causing birth defects in rabbits. Two junior researchers who worked on the study claimed McBride adulterated the results before they were submitted to the *Australian Journal of Biological Sciences*.

Dr Norman Swan of the Australian Broadcasting Corporation's science unit, who first broadcast the accusations against McBride, says he is happy with the composition of the committee, but concerned that its deliberations will be secret. He also has reservations about its scope — it will not look into the larger question of how science has been administered by Foundation 41, particularly the lack of a properly operating peer review mechanism. Nor will it consider the question of compensation for the junior researchers who first "blew the whistle" and whose careers have suffered as a consequence.

Submissions to the inquiry can be made in writing to S. R. Weedon, Secretary to the Committee, PO Box 510, Woolahra, NSW, 2025, Australia. Charles Morgan

First drought and next a plague of extremely unfriendly bees

Washington

THE last opportunity to stop the spread into the United States of the Africanized honey bee — dubbed the “killer” bee by the popular press — appears to have been lost. Hastily conceived plans to halt the bees in Mexico are suffering from a lack of organization and seem doomed to failure.

The result could be devastating for the US bee-keeping industry, with a major impact on the \$20,000 million annual production of bee-pollinated crops.

Africanized honey bees have been spreading north from Brazil at a rate of 200–300 miles a year since they were accidentally released in a breeding experiment there in 1956. They have now reached southern Mexico.

The bees are the progeny of a cross between the African and the European races of the honey bee, *Apis mellifera*, intended to create a race of bee ideally suited to tropical honey production. But European bees have been domesticated for centuries and are mild-tempered and easy to keep. Unfortunately, Africanized bees are very different and defend their hives aggressively. Hives of European bees can be placed out in orchards and fields without threat to passers-by. Africanized bees defend an area within about a quarter mile of their hives and will mount spectacular mass attacks on intruders.

Thirty to forty stings per square inch of exposed skin have commonly been recorded from victims of bees. Many people and animals have died as a result.

Africanized honey bees are also poor honey producers and swarm readily, leaving their hives to set up feral nests. Once in the wild they are highly successful, out-competing native wild bees and domestic European honey bees in gathering nectar.

As the Africanized honey bees have spread from Brazil, they have interbred with European honey bee populations. But that has failed to dilute out their unfavourable characteristics — most probably because the Africanized bee population grew rapidly in the wild and the populations of domestic bees are small in South and Central America.

The US Department of Agriculture (USDA), working in collaboration with the Mexican Ministry of Agriculture, planned to halt the bees at the Gulf of Tehuantepec, Mexico's narrowest point. The idea was to dilute rapidly the African genes in the population by destroying feral bee nests and flooding the area with European drones. But the funding and organization came too late, and the Africanized bees had already arrived. The defence zone had to be pulled back north,

to two coastal lowland corridors either side of the Mexican plateau.

Jose Villa, who is helping to coordinate the efforts of USDA and the Mexican Minister of Agriculture, says he “still maintains hope” that enough European bees will be in place to counteract the dense migration of Africanized bees already occurring a few hundred kilometres to the south. But he says that there are organizational problems and plans to saturate the area with drones are behind schedule. Villa notes that Congress appropriated only a fraction of the funds requested, but says that lack of time is a bigger problem than lack of money.

Norman Gary, professor of entomology at University of California, Davis, thinks there is no longer much chance of stalling the Africanized bees: “It's like fighting a



fire, when you are fighting in one area, its spreading in another”, he says.

US bee-keepers will face a suite of problems once the Africanized bees arrive. Their best hope is continually to re-queen hives whose queens have mated with Africanized drones. But even specialists cannot tell Africanized and European bees apart without a careful microscopic examination. And it will be difficult to maintain a stock of mated replacement queens which are known to have come from unaffected areas.

Litigation may be another problem for the commercial bee industry. Lawsuits are sure to arise if hives containing Africanized bees cause a fatality. Richard Hellmich, a research entomologist at the USDA research service at Baton Rouge, dreads a public outcry, feeling that “legislation and over-reaction may damage the bee industry more than the Africanized bees”. Hellmich thinks there may be no way to get rid of the Africanized bees, but that vigilant bee-keepers will be able to cope. He points out that the Venezuelan bee industry collapsed after Africanized bees arrived, but has since managed to recover. “We might have to learn to live with them as we do with cockroaches”, Hellmich says.

Alun Anderson

NERC's cuts cost more jobs

London

THE long-standing financial difficulties of the Natural Environment Research Council (NERC) came to a head last week with the announcement of 130 redundancies to take effect before next April. The research areas to suffer were due to be announced early this week. Marine sciences and terrestrial ecology are to be hardest hit, according to the Institute of Professional Civil Servants (IPCS), which represents public sector researchers. The research station of the Institute of Oceanographic Sciences in Wormley, Surrey, is expected to lose half of its 200 staff; the research station in Wales of the Institute of Terrestrial Ecology could lose around half of its 27 researchers.

NERC has suffered a cash crisis over the past three years because of declining government support. NERC's science budget allocation has declined in real value by £2.5 million and income from research commissioned by government departments has decreased by £4.5 million. The increase in commercial income has not compensated for this, so the council was forced earlier this year to cut its support for university research by £2.6 million and to cancel the grant round for 1988. It has now been allocated £3 million for shedding staff by the Advisory Board for the Research Councils. The latest staff cuts are part of the trend which saw a reduction of 16 per cent in the staff at NERC between 1983 and 1987.

Meanwhile, financial pressure on Britain's Agricultural and Food Research Council (AFRC) has hit research into animal health. Research at the Institute for Animal Health's laboratories at Compton, Houghton and Pirbright is to be concentrated at the Compton laboratory. Houghton, which carries out research into poultry diseases, is to close, with half the 170 staff fearing redundancy. The rest will be moved to Compton. The situation at Pirbright is still unclear, though research into exotic diseases will continue there.

Since 1983, AFRC has shed around 25 per cent of its workforce, which now stands at almost 5,000, and numbers are expected to decrease further next year. Research into animal health has taken an uneven share of the cuts, with a reduction in the institute's staff of 40 per cent to the present 500.

Government policy calls for near-market work in current public research programmes to be transferred to industry funding. The IPCS fears the government is now looking for large cuts in agricultural research following a Ministry of Agriculture review of near-market research

Christine McGourty

Dealing with job applicants

SIR—At each rung of the academic ladder, an aspirant can expect to face a search committee. The meeting of applicant with evaluators is likely to be stressful under the best conditions, but the manner in which a search committee operates can make a difference. My recent experience as a job seeker suggests that many committees could use a lesson in etiquette, so, in the absence of a scientific Miss Manners, I propose to offer advice to those who will serve on search committees.

1. *Acknowledge receipt of applications.* Committee members may feel overwhelmed by hundreds of applications, but should remember that each application represents a large amount of work by the applicant and deserves a polite response. Fifteen per cent of my applications were never acknowledged. After a while, I began to think of the mailbox as a black hole. Upon receipt of an application, a search committee should write to thank the applicant for her or his interest in the job and to provide a tentative timetable for the search.

2. *Keep applicants informed.* As decisions are reached, applicants should immediately be told if they have been rejected, placed on a short list, or will be interviewed. About 30 per cent of the search committees never told me how I stood in the search, others took as long as 15 months to reply, and some even failed to tell me how a search was concluded after I was interviewed. I suggest that applicants should be contacted every two to three months until their status is final.

3. *Don't run a search for a job that doesn't exist.* This rule may seem obvious, but 5 per cent of the jobs for which I applied and even one for which I interviewed were cancelled by central administrators. Besides wasting everyone's time, such cancellations suggest to a large number of qualified applicants that your organization is in disarray. If you must cancel a position, a humble letter in which the cancellation is fully explained and blame is accepted can mitigate the damage.

4. *The interview should be well-organized and should consider the wishes of the candidate.* Most of my interviews were well run and left me with positive impressions, but many interviews do more harm than good. I know candidates who have been stranded at airports and housed at unsafe hotels, who have arrived without appointments or seminars scheduled, who have been taken to restaurants where they could not eat because of dietary needs, and who were neglected for long periods. A committee should appoint a member to set up the candidate's schedule and coordinate the interview, and this person should consult with the candidate before the interview to set up transportation and

lodging, to find out if the candidate wishes to speak with particular people or visit particular parts of campus, and to determine if the candidate has other specific needs.

5. *Don't insult the candidate.* I suppose the few search committees deliberately attempt to insult candidates, so it is surprising how often it happens. It was depressing to be confronted with a department chairperson who would not give straight answers to questions about laboratory space, set-up money, teaching or other questions. These instances were indirectly insulting, but it was much worse to be confronted with racist or sexist remarks. Most of my interviews included at least one meeting where derogatory comments about minority groups or women scientists were made. Imagine my dismay when one male scientist confided that the woman in a scientific couple seldom amounted to much. The man who said this is on a faculty with married scientists, is married to a scientist, knew that I am married to a scientist, knew that I trained with a woman scientist, and knew that my thesis adviser's wife is in the National Academy of Sciences! Other examples were equally bad, and each such statement suggested to me that I would be better off somewhere else. (In retrospect, I should perhaps be grateful that so many people were so guileless as to reveal their true thoughts in a short discussion.)

Why should a search committee bother to follow these suggestions? After all, when you gaze at those hundreds of applications, you may think that it will be no trouble to attract your top choice. Consider, however, that stories of poorly run searches and interviews rapidly circulate among potential job candidates and can make them wary of your department. Consider that, even in the current job market, most people receive more than one job offer so you will probably be competing for your top choice. Finally, consider that many of the people who apply to you will be around for years to come, and could come back to haunt you in unexpected ways. In my experience, search committees that pay attention to etiquette are run in exemplary fashion, and leave everyone involved satisfied that a difficult task has been well performed.

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Spanish science

SIR—The National Plan for Scientific Research and Technological Development is in the process of being precisely defined in Spain. The government has considerably

increased economic support for research, in particular in grants for several priority fields. But there are structural problems that are not being tackled, such as the inadequacy in the national distribution of research centres or the comparatively low salaries of scientists.

The national plan defines objectives according to which grants are available. But there seems to be no plan to provide new positions. The number of scientists in temporary postdoctoral positions both in Spain and abroad is increasing alarmingly. The situation is most acute for those scientists who have worked abroad for many years and want to establish their own research projects upon returning to Spain. Many of them have obtained permanent positions in the Spanish research council (CSIC), but when they want to start working in one of its institutes, little space is available and their access to research funds is severely limited. Scientists returning home are not warmly received by the CSIC and many choose in the end not to return under such conditions.

It seems to me that more scientists and more laboratories should be involved in the development of the national plan. Some time ago, Dr E. Trillas, president of the CSIC, declared in an interview that "one may put obstacles in the way of outstanding scientists, because they will go ahead anyway". This is of course true, but it will not help scientists to become established in Spain.

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Who should pay?

SIR—It seems to me that the essential question about who is paying for new drugs is not "Is it acceptable for patients to fund research indirectly but not acceptable when the direct relationship is apparent?" as suggested by Robert Oldham (*Nature* 332, 795; 1988) but whether it is an improvement that a patient is charged a large amount of money for treatment, whereas we invented health insurances to share costs that cannot be borne by one person. The answer has to deal not only with ethical aspects but also with the rate of progress that can be made in clinical research using direct or indirect charges. I therefore support Oldham's conclusion that "the question of who pays for clinical research needs broad public debate and openness on the part of all the parties involved in developmental therapeutics".

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Turning phases into frequencies

The concept of geometrical phase, hitherto largely an academic issue, seems unexpectedly to have led to a novel means of fine-tuning the frequency of a laser.

It cannot be often that brooding about the basis of quantum mechanics leads people to the invention of a practical device, but this is what appears to have happened to R. Simon (from the Institute of Mathematical Sciences at Madras) and H. J. Kimble and E.C.G. Sudarshan from the University of Texas at Austin. They have been seeking a more general understanding of 'Berry's phase', or geometrical phase as M. V. Berry from the University of Bristol would modestly prefer it to be known, and have hit on a scheme for fine-tuning the frequency of a laser (*Phys. Rev. Lett.* **61**, 19; 1988).

The confusions surrounding this issue have very little to do with quantum mechanics, but are instead bound up with the shadowy character of the concept of phase. This is simply a consequence of the fact that phase as such is hardly ever a measured quantity, a number. It is true, of course, that every interference experiment is an implicit measurement of phase. The bands of illumination on a screen behind a pair of Young's slits illuminated with monochromatic light are nothing but measures of the relative phase of the radiation arriving by two different routes.

If the result were not so visually spectacular, people might find themselves actually measuring phases and subtracting them from one another. But, in the event, phase usually makes its appearance as an apparently adjustable parameter in, for example, the equation of a wave; secondary-school students can write down $\cos(\omega t + \phi)$ as readily as anybody, but ϕ is rarely endowed with physical significance.

That state of affairs can be readily changed. Berry himself (see *Nature* **326**, 277; 1987) has given a neat account of the transport of polarized light along a coiled optical fibre, showing that the plane of polarization of a propagating light wave is rotated by an amount determined by the solid angle contained within the cone-shaped object, generated by anchoring to the same origin all the unit vectors representing the direction of the optical fibre. To put it another way, circularly polarized light waves are shifted in phase, but the phase shifts are in opposite directions for right- and left-handed polarization. All this, says Berry, is strictly classical.

On the face of things, phase arises in quantum mechanics in exactly the same way as in elementary descriptions of waves, but there is a complication because

of the way in which a wave function can be multiplied by any number of unit modulus (the number will in general be complex) without affecting its physical significance (because all that matters is the product of the wave function and its complex conjugate, which is the probability of finding a particle at some point in space).

This leads to two important notions, first that of gauge invariance as in quantum electrodynamics and, second, the Aharonov-Bohm effect by which the state of a wave function is affected by electromagnetic potentials that do not generate electric or magnetic forces on the system as such. The neatest illustration is that of a Young's slit interference system for electrons with a long thin solenoid carrying a current placed symmetrically between the slits; the magnetic forces can be made indefinitely small by making the solenoid longer, but the electromagnetic potential (normally unobservable) remains unchanged, so that the interference pattern is distorted.

Berry's version of the geometrical phase is not so much a generalization as an independent but similar observation of what will happen to a quantum system if some parameter involved in its definition is slowly changed, which may occur for a host of reasons. The varying parameter may, for example, represent the strength of some externally imposed field or even the strength of the interaction between one part of the system and the rest. If the varying parameter eventually returns to its original value, so too will the quantum system return to its original state, but the phase will in general be different, and again be determined by a solid angle measuring the expanse of the track of the changing parameter in some kind of parameter space. Presumably what this means is that the state of a system, although not its actual physical properties, is marked by its recent history.

During the past year, several people have sought further generalizations of this notion. One of these is the generalization due to Aharonov and Anandan which, as Simon and his colleagues put it, "liberates" the geometric phase from the requirement that changes of the controlling parameter should be slow by the standards of the timescales characteristic of the system itself. Their trick was simply to recognize that the state of a quantum system can be represented as a vector in the infinite space of an acceptable set of

eigenfunctions, and that the vector must trace out some closed circuit whose characteristics can be related to the externally imposed changes, whether quick or slow.

How does this relate to the fine-tuning of the frequency of a laser? This is how the argument goes. First, you construct a kind of Michelson's interferometer with input from a helium-neon laser. Use a beam-splitter to direct the laser beam into two orthogonal tracks, with a mirror at each end, and use one of the arms as a means of measuring the phase of the light beam in the other arm. Then arrange that the input source of light is plane polarized in a known direction, most simply by interposing a half-wave plate. Then the fun begins.

The business arm of the interferometer must be fitted with two quarter-wave plates, the first of which is set with its slow axis at an angle of 45° to the plane of polarization. The output from that device is circularly polarized light, which is then channelled through a second quarter-wave plate rotating slowly and at a constant rate on its axis. Because of the mirror at the end of the arm, the light beam of course makes two circuits of the course before interfering (or not, as the case may be) with the reference beam.

Simon and his colleagues note that this is essentially a system with quantum states — the states of polarization — which is influenced by an externally varied parameter, the position angle of the second quarter-wave plate which controls the state of polarization of the light beam in the several sections of the business arms of the interferometer. The argument quickly leads to the conclusion that the frequency of the light output from the interferometer is shifted by an amount determined by the rate of rotation of the quarter-wave plate.

That there should be a connection between phase and frequency is not surprising; both, after all, turn up together in any formula that describes a wave. But on this occasion, the usual phenomenon of beating does not occur. The real interest of the exercise, as conducted by Simon and his colleagues, is their use of a neat way of representing states of polarization as points on the surface of a sphere (originally devised by Poincaré, as it happens) which is also a neat way of getting to grips with the SU(2) group, famed for its role in the electro-weak interaction.

John Maddox

Control of cell growth

Molecular associations and conceptual connections

Miranda Robertson

THE choice of intracellular signal transduction as the topic for this year's Cold Spring Harbor Symposium* was inspired by the hope that it would be possible to make the connection between the binding of a signal to its receptor on the surface of a cell and the transcriptional activation (or repression) of specific genes in the nucleus. This aspiration was not quite met; but important connections were nonetheless made, in particular in the chain of events by which extracellular signals control cell growth.

Most notably, a connection in the literal sense of the word has been established between the product of the *retinoblastoma* (*Rb*) locus, implicated in some human cancers, and the oncogenic products of two DNA viruses (see pages 124 and 168 in this issue^{1,2}); and the protein kinase encoded by the yeast cell-cycle gene *cdc2* and *cdc28* seems to be the yeast homologue of the vertebrate maturation-promoting factor (MPF) which is essential for the entry of mammalian cells into mitosis (see box). Moreover, although there is no single case in which a complete chain of reactions between cell surface and nucleus has been defined (with the exception of that of the steroid receptors, which I shall not discuss here), it is now possible to see at least in outline how the activation of a growth-factor receptor could, by modification of pre-existing cellular proteins, rapidly activate genes whose products in turn induce cellular proliferation.

Suppressing the suppressors

The genes whose induction stimulates cellular proliferation include many that have been identified as the cellular counterparts of dominantly acting retroviral oncogenes. The link, so far quite circumstantial, between the *Rb* product and the DNA tumour viruses is of a rather different kind. The retinoblastoma gene is one of a growing number implicated in rare familial cancers in which the inherited deletion or disablement of one copy of the gene confers a strong predisposition to the development of specific tumours — in the case of the *Rb* gene, retinoblastomas and osteosarcomas. Because the tumour cells of patients have invariably lost the remaining normal copy of the gene, it has been inferred that the *Rb* product normally suppresses cellular proliferation.

It now seems possible that the transforming DNA viruses may relieve this suppression by titrating the *Rb* product: Ed Harlow (Cold Spring Harbor Laboratory; ref. 1) and David Livingston (Dana-Farber Institute, Boston) report that a cellular protein with a relative molecular mass of 105,000 (105K) found in association with the E1A protein of adenovirus and which binds to an analogous region of the SV40 large-T antigen, is the product of the *Rb* gene.

The precise significance of this association is unknown. The *Rb* gene product is a nuclear phosphoprotein that binds DNA-cellulose columns³ but has not yet been identified with any specific DNA-binding activity that might indicate a direct role in gene regulation. There is however indirect evidence suggesting that the binding of the *Rb* protein by viral transforming proteins may affect the regulation of the cell cycle.

Detailed mutational analysis of the E1A protein^{4,5} has identified a region (region 2) that is required for transformation and in particular seems to be necessary to drive cells into mitosis. Peter Whyte, in Harlow's laboratory, has shown that deletions in region 2 reduce or abolish the binding of the 105K protein now shown to be the *Rb* gene product; and Elizabeth Moran⁶ has shown that the transforming activity of region 2 deletion mutants of E1A can be restored by replacement of the missing region 2 with a homologous region⁶ of the SV40 large-T antigen.

Although the mechanism of the transforming activity of region 2 of E1A and SV40 large-T antigen is unknown in detail, there is suggestive biochemical as well as genetic evidence for a role in control of the cell cycle: sequences in both these proteins are the substrate for casein kinase II, a serine-threonine kinase that is activated by growth factors⁷ and can be shown to phosphorylate peptides derived from domain 2 of the viral oncogenes, as well as from cellular oncogenes *c-myc*, *c-fos* and *c-myb*, in a cell-cycle-dependent manner (Denis Carroll, Cold Spring Harbor Laboratory).

A further tenuous link with human cancer is forged by the discovery of Phelps *et al.*⁸ that papillomavirus, which is strongly implicated in cervical cancer, also has a region homologous to region 2 of E1A.

Activating the activators

Except in the case of the steroid receptors, where the receptor for extracellular signals is also the nuclear transactivator,

the binding of cell-surface receptors by signalling molecules activates gene-regulatory proteins indirectly, through intermediary reactions involving protein kinases. Representatives of several components of these signalling pathways have been identified as the products of cellular counterparts of retroviral oncogenes. The nuclear proteins identified in this way occupy a pivotal position on the transduction pathway. These proteins, which include the products of *c-myc*, *c-fos* and *c-jun*, are among the so-called immediate early proteins of the proliferative response: that is, they are induced rapidly by growth factors in the absence of new protein synthesis and are believed to activate in turn the genes necessary for cell division (Daniel Nathans, Johns Hopkins University; Rodrigo Bravo, European Molecular Biology Laboratory, Heidelberg).

In a complementary (c)DNA library constructed by Bravo from fibroblasts stimulated by growth factors, 81 immediate early sequences have already been identified and there may be as many more. This shotgun approach to the identification of immediate early genes has yielded not only the known *myc*, *jun* and *fos* genes but at least one further sequence related to *jun*⁹ and one to *fos*¹⁰.

The transcriptional activation of the immediate early genes must be under the control of transactivating proteins that are constitutively present in the cell and may be the targets of the kinases activated by the binding of growth factors at the cell surface. Of the pivotal nuclear c-oncogenes the most rapidly induced is *c-fos*, which is activated within 5 minutes of stimulation by growth factors¹¹; and because *c-fos* expression is controlled principally at the transcriptional level, it has been a particularly attractive starting-point for research on the mechanisms of activation of the immediate early genes. At the same time, interest in the effector functions of FOS has been sharply boosted by the discovery of its association with the product of a second immediate early gene, *c-jun*^{12,13}, now known to be the transcriptional regulator AP-1^{14,15}.

Mechanisms of activation

In the mouse fibroblasts in which the induction of *c-fos* has principally been investigated, the gene is responsive to growth factors and phorbol-ester tumour promoters, both acting through the serum-responsive element (SRE) a palindromic sequence which because of its dyad symmetry is sometimes known as the dyad symmetric element (DSE)^{16,18}. This sequence is defined as the binding site for a ubiquitous nuclear phosphoprotein known as serum responsive factor (SRF) which is presumed to mediate in some way the activation of *c-fos* in response to serum (or growth factors contained in it). The

*The Cold Spring Harbor Symposium on The Molecular Biology of Signal Transduction was held at Cold Spring Harbor on May 25–June 1, 1988.

mechanism whereby SRF is activated by growth factors is unknown, but there are three ways in which it might work in principle. First, phosphorylation by growth-factor-activated kinases might induce DNA binding by promoting dimerization where the monomeric protein has only weak affinity for its binding site. This is very unlikely to apply to SRF which seems to dimerize without stimulation. Second, it might increase the ability of the protein to activate transcription without affecting its affinity for DNA. Third, it might promote the formation of a complex between the SRF dimer and a separate protein containing or comprising a transcriptional activating domain. This might seem a somewhat gratuitous suggestion but for some recent evidence, to which I shall return later, for the formation of functionally important complexes between at least some of the immediate early gene products themselves. The isolation by Triesman of the gene encoding SRF should accelerate the exploration of this and other possible modes of activation of the SRF protein.

Evidence for a contribution of at least two of these mechanisms to *c-fos* activation may be more rapidly forthcoming from investigations on its response to cyclic AMP, which induces *c-fos* transcrip-

tion through a pathway that is independent of the serum-responsive element and is presumed to depend upon one or more of the sequences corresponding to defined cyclic-AMP-responsive elements (CREs) in the regulatory region of the gene (Ron Prywes, Rockefeller University; Inder Verma, Salk Institute; Michael Gilman, Cold Spring Harbor Laboratory).

Direct evidence for this pathway has been adduced from experiments by Karl Riabowol with Michael Gilman in James Feramisco's laboratory (Cold Spring Harbor Laboratory). Microinjection into cells of the catalytic subunit of the cyclic-AMP-dependent protein kinase A leads to the activation of *c-fos* transcription, and the effect can be blocked by the co-injection of oligonucleotides corresponding to a cyclic-AMP-responsive element but not by a serum-responsive-element oligonucleotide. Investigations by Yamamoto *et al.*¹⁹ suggest how protein kinase A activated by cyclic AMP might activate proteins binding to cyclic-AMP-responsive elements (CREB) and reveal a complex picture of their regulation.

Using a CREB from brain, Verma and Yamamoto *et al.* have shown in gel-retardation assays that whereas the 43K brain protein will bind to its cognate element in either the *c-fos* or the somatostatin gene, a

higher molecular weight complex (presumed to be a CREB dimer) binds with higher affinity. Experiments with protein kinases *in vitro* have shown that phosphorylation of the purified protein at two sites promotes dimerization, whereas phosphorylation at a third, separate site increases the ability of the protein to activate transcription. Only the increase in transcriptional activation is, however, attributable to phosphorylation by protein kinase A: unexpectedly, it is phosphorylation by protein kinase C — not normally associated with cyclic AMP-mediated signalling — that promotes dimerization. While most of these experiments were done with the somatostatin CRE, it seems likely that similar mechanisms apply to the equivalent element in the *c-fos* gene.

The regulatory region of *c-fos* is complex and contains, as well as binding sites for serum-responsive and cyclic AMP-dependent factors, at least one sequence corresponding to the specific binding site for AP-1, a factor now implicated in the effector functions of FOS. One of these is the regulation of its own production; and at Cold Spring Harbor two groups reported evidence implicating AP-1 in the mechanism of this auto-regulation.

Suppressing the activators

It is a feature common to the immediate early genes that their activation is not only rapid but transient; and the enhanced transcriptional activation of genes such as *c-myc* and *c-fos* under the inhibition of protein synthesis led very early on to the suggestion that they negatively regulate their own expression as well as (presumably) positively regulating the expression of other cellular genes. (It is this negative feedback that is believed often to be abrogated or overridden in oncogenic mutants at least of *c-myc*.)

Neither MYC nor FOS however has ever been shown to bind specifically to DNA, and their mechanism of action remained moot until recently when it transpired that the 39K protein with which FOS is commonly associated is the mammalian transcription factor AP-1, which is the product of *c-jun*^{12,13,20}. The revelations that have followed this discovery apply in practice only to the function of FOS, but there are indirect suggestions of a similar role for MYC and other products of nuclear cellular oncogenes such as *c-myc*.

Like *c-fos*, *c-jun* is rapidly induced by growth factors; in the absence of FOS, JUN binds weakly but specifically to the DNA binding site defined by its ability to bind AP-1; in the absence of JUN, FOS does not bind the AP-1 site at all. When both are present, FOS is found to associate with DNA containing the AP-1 binding site, and the specific binding affinity of JUN is increased (Tom Curran, Roche Institute, New Jersey; Robert Tjian, Berkeley). This suggests that FOS and

From egg to ascus

Maturation-promoting factor (MPF) was first discovered as a cytoplasmic extract of mature *Xenopus* eggs that would, on injection into immature oocytes, induce entry into meiosis. It has since been demonstrated in the cytoplasm of both yeast and mammalian cells where it induces mitosis in the normal cell cycle. Although it was discovered in 1971, and has been known for some years to be a crucial component of cell-cycle regulation in mammalian cells, it has proved extraordinarily resistant to purification and its nature and mechanism of action have eluded precise definition.

There is thus an element almost of bathos in the evidence presented by David Beach (Cold Spring Harbor Laboratories) that MPF, or at least an essential component thereof, is a 34K protein kinase identified last year^{1,2} as the product of the mammalian homologue of the cell-cycle gene *cdc2* of fission yeast. The story is as follows.

With Giulio Draetta, Beach has shown that the 34K protein kinase (p34) of HeLa cells exists in an inactive complex with a second protein, p13, until late in the G2 phase of the cell cycle, when its phosphorylation is increased and it becomes associated with a third protein (p62); at this stage it is activated, its kinase activity rising to a peak at the point of entry into mitosis. It thus shares with MPF the property of increased kinase activity without any increase in abundance.

The evidence for identity with MPF

comes from investigations, done in collaboration with William Dunphy and John Newport, into the effect of the yeast p13 protein on the action of MPF on mitotic initiation in cell-free extracts of *Xenopus* eggs. It has been impossible to demonstrate directly that the product of the *cdc2* gene can promote entry of *Xenopus* nuclei into mitosis, but Beach reasons that this may be attributable to some failure to reproduce the appropriate phosphorylation and subunit composition with the heterologous protein. What he has now shown is that p13 added to cell-free extracts will block the effect of *Xenopus* MPF, partially purified by Lohka *et al.*³, and that MPF activity is retained by a p13 column, which moreover retains the *Xenopus cdc2* product, along with a 42K protein that may be a second component of *Xenopus* MPF.

How far yeast cell-cycle genetics will now be able to clarify the control of proliferation in mammalian cells remains to be seen. Although the mammalian *cdc2* product will complement temperature-sensitive yeast mutants, this conservation of biochemical function does not necessarily reflect a precise conservation of physiological function: *cdc2* in fission yeast seems to be required in G1 and G2 rather than at mitosis; and the budding-yeast homologue, *cdc28*, controls entry into G1.

1. Draetta, G., Brizuela, L., Potashkin, J. & Beach, D. *Cell* 50, 319–325 (1987).

2. Lee, M. & Nurse, P. *Nature* 327, 31–35 (1987).

3. Lohka, M.L., Hayes, M.K. & Maller, J.L. *Proc. natn. Acad. Sci. U.S.A.* 85, 3009–3013 (1988).

JUN form a DNA-binding complex, although it is not clear whether FOS promotes the formation of a JUN dimer (the JUN binding site has dyad symmetry) or whether FOS itself forms a DNA-binding heterodimer with JUN.

As the structural substrate for the FOS–JUN association, Curran invokes the leucine zipper, a device postulated by Landschulz *et al.*²¹ to facilitate the formation of DNA-binding complexes between proteins with a common leucine-rich motif. These include MYC, FOS, JUN and the yeast transcriptional activator GCN4, as well as CEBP, an enhancer-binding protein recently characterized in Steven McKnight's laboratory.

The two halves of the leucine zipper are formed by sequences predicted to fold as alpha helices with leucine residues, which occur at every seventh residue for a distance of eight helical turns, along one side; the bulky side-chains of the leucines on the surface of one protein being presumed to interdigitate with those on another to stabilize complex formation. Landschulz *et al.* suggest that this interaction could align two weakly DNA-binding regions in the two halves of a dimer in such a way as to generate a high-affinity DNA-binding complex.

Although there is no evidence at this stage for this interesting suggestion, the existence of the leucine motif in so many nuclear proteins of related function makes it extremely compelling; and recent genetic evidence on the organization of enhancer elements²² lends it extra appeal.

Whatever the nature of the complexes formed by FOS and JUN, there is now clear evidence that the association is functionally significant. Both Verma and Tony Hunter (Salk Institute, with Michael Karin, University of California, San Diego) have shown that a reporter gene

containing an AP-1 binding site can be strongly activated in F9 cells, in which endogenous levels of AP-1 are negligible, only if they are co-transfected with expression vectors for both FOS and JUN. Similarly, in a series of experiments with a reporter gene containing the AP-1-binding tumour-promoter-responsive element in NIH 3T3 cells, Axel Schöntal (Institute of Genetics and Toxicology, Karlsruhe) has shown that co-transfection with *fos* and *jun* expression vectors strongly activates the reporter gene, and the effect can be abrogated by cotransfection with expression vectors containing either *fos* or *jun* antisense sequences.

Moreover, Verma and Schöntal have extended their observations to show that FOS and JUN will also cooperate to repress *c-fos* transcription from its own promoter. Thus the evidence from these test systems suggests that the association between FOS and JUN is the structural basis for the responsiveness of some AP-1 binding sites to stimulation by tumour promoters. The relationship of the upstream AP-1 site of *c-fos* to negative autoregulation is more problematic, since transcriptional repression by FOS survives the deletion of the AP-1 site (Michael Gilman, Ron Prywes). Robert

Franza (Cold Spring Harbor Laboratory) however reported that FOS complexes will bind the cyclic-AMP-responsive element; which may introduce a further element of complexity but at least provides a possible alternative substrate for autoregulation.

Transcriptional repression is exceedingly ill understood, but there is at least one precedent for a transcription complex that, like FOS–JUN, can both activate and repress. In their paper describing the complex, which participates in the control of cell-type-specific genes in yeast, Keleher *et al.*²³ suggest two possible mechanisms for repression: the masking of an activator protein, or very high-affinity binding of essential components of the transcriptional machinery so as to lock them in place. If either is applicable to cell-cycle regulation by FOS–JUN, this would suggest the need for further regulatory mechanisms by which *c-fos* transcription might be released from repression. Some such additional level of control might be afforded by the phosphorylation known to occur at several sites on both FOS and JUN, with unknown effects on function. □

Miranda Robertson is Biology Editor of Nature.

Polar motion

Blowing in the wind

Thomas A. Herring

It now seems that most fluctuations of the Earth's rotation with periods less than a year are caused by motions of the atmosphere. This relationship has been postulated for some time and it was already known that short-period variations in the rotation rate of the Earth can be accounted for by changes in the polar component of the angular momentum of the atmosphere (Carter, W.E. *et al. Science* **224**, 957–961; 1984 and Morgan, P.J., King, R.W. & Shapiro, I.I. *J. geophys. Res.* **90**, 12645–12652; 1985). Now T.M. Eubanks *et al.* show elsewhere in this issue (*Nature* **334**, 115–119; 1988) that short-period wobbles of the rotation axis (polar motion) are mostly attributable to changes in the equatorial component of the angular momentum of the atmosphere. This raises questions about the effect of the oceans and earthquakes on the Earth's rotation. Moreover, there remain questions about the origins of long-period changes.

The importance of the atmosphere in changing the rotation of the Earth has been suspected for a long time. The annual effect of the atmosphere on polar motion had been anticipated by Kelvin as early as 1862. But only in the past decade have measurements of polar motion and

meteorological data become sufficiently accurate for the week-to-week influences of the atmosphere on polar motion to be studied. The improved accuracy of the measurements of polar motion arise from the use of very-long-baseline interferometry (VLBI) — the precise observation of extragalactic radio sources (see the News and Views article by Peter Scheuer on page 107 of this issue) — and satellite laser ranging (SLR), the measurement of the distance to satellites orbiting the Earth. Each technique can determine where the rotation axis pierces the Earth's surface with a precision of about 5 centimetres. Both are routinely used to monitor the rotation of the Earth and form the backbone of the new International Earth Rotation Service. They are also used to study plate motions, regional tectonics in Alaska, California and the Mediterranean, changes in the Earth's gravity field and the flattening of the core–mantle boundary.

Polar motion is a combination of a free motion with a natural period of about 435 days (Chandler wobble) and forced motions, which are dominated by an annual variation of atmospheric origin but also have large (more than 1 metre) short-period fluctuations. The study by Eubanks *et al.* reported in this issue

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largely resolves the origin of the excitation of the short-period polar motions, although some questions remain. The changes in atmospheric angular momentum associated with mass redistribution in the atmosphere, as measured by changes in surface pressure, seem to be determined accurately by the various meteorological services and correlate well with the oscillations. But the effects of the pressure changes over the ocean and the contributions of global winds to the angular momentum are still debated.

If the oceans were in equilibrium for forcings with periods of 20–50 days, there would be little change in pressure on the sea floor: the ocean would flow in response to the pressure changes rather than load the sea floor, and so would not cause any change to the Earth's rotation. Studies of the ocean tides show that the diurnal response of the ocean is far from equilibrium. For periods greater than a year, the response is thought to be within a few per cent of equilibrium. Polar-motion studies suggest that in the intermediate range of periods some parts of the ocean have an equilibrium response but that other parts, particularly the southern oceans, are far from equilibrium.

The uncertainty about the contribution of the winds arises mainly from the accuracy with which the global wind patterns can be determined by the meteorological services. The results obtained by different services disagree considerably even though they largely use common data, because the services use different schemes to predict the wind at places for which there are no data. The winds and non-equilibrium responses of the oceans, however, seem to be sufficient to account for the remaining short-period polar motion.

Despite the progress made in understanding polar motion, there remains one significant unanswered question. What is the excitation of the Chandler wobble? This free motion has an average amplitude of about 4.5 metres, although this amplitude has varied by as much as a factor of two during this century. The decay time is thought to be between 10 and 20 years, but it could be greater. No one has yet conclusively demonstrated an excitation mechanism that could power the free motion. With the combination of the new accurate geodetic techniques and accurate modelling of the atmospheric forcing, it will be possible to study more directly the free mode by removing all of the known forced motions. With the atmospheric excitation removed, it might be possible to see if there are earthquake excitations of the Chandler wobble, and, if there are, even to elucidate the mechanisms involved in these catastrophic events. □

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Neuronal development

Not all about *eve*

Haig Keshishian

NERVOUS systems are the most complex biological structures known — even in invertebrates a dizzying array of structurally unique neurons are generated during embryogenesis. Which genes must be active during development to specify a neuron's characteristic identity? What has emerged from several recent studies of fruitflies^{1–4} (*Drosophila*) and nematode worms⁵ is that genes containing homoeo boxes can be important in the cellular determination of neurons.

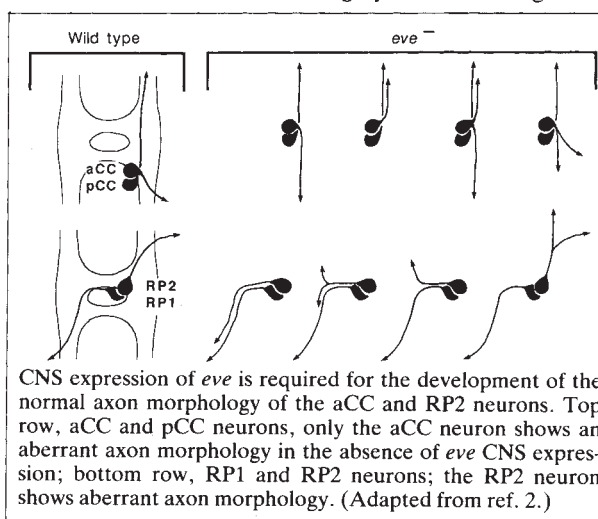
Homoeo boxes encode a highly con-

the homoeotic genes of the Antennapedia and Bithorax complexes). Although *eve* and *ftz* proteins are expressed in the blastoderm in the well-known pattern of seven circumferential zebra stripes, the proteins are later expressed again in specific neurons of every segment. What role do the proteins play during their encore appearance?

By dye-filling the tiny *Drosophila* neurons intracellularly with Lucifer yellow, Doe *et al.*² examined their anatomy following the suppression of *eve* expression.

In each pair, the cells develop into neurons with distinct axonal trajectories (see figure). In the absence of *eve* expression, however, one neuron in each pair grows normally while its sister projects its axon incorrectly. In the RP1–RP2 pair, for example, only RP2 expresses *eve*. When expression is blocked, the RP2 neuron is usually transformed anatomically towards RP1, whereas RP1 remains normal.

The transformation of RP2 towards RP1 when *eve* expression is blocked is reminiscent of the 'fate-



CNS expression of *eve* is required for the development of the normal axon morphology of the aCC and RP2 neurons. Top row, aCC and pCC neurons; only the aCC neuron shows an aberrant axon morphology in the absence of *eve* CNS expression; bottom row, RP1 and RP2 neurons; the RP2 neuron shows aberrant axon morphology. (Adapted from ref. 2.)

served sequence of 60 amino-acid residues (the homoeo domain of the protein). The homoeo domain probably binds a site on the DNA of its target gene and thereby regulates RNA transcription. Antibodies against proteins with homoeo domains invariably label the nucleus. At least two dozen genes with homoeo boxes have been found in *Drosophila*, and most metazoans, including humans, have them. At first, it seemed that the role of these genes in *Drosophila* was limited to pattern formation and segmentation in the early embryo. But as the roster of homoeobox-encoding genes has grown, new developmental roles have been proposed. Some of the genes are expressed at times well after the embryo has become segmented, and the late-appearing products can show up in specific neurons of the central and peripheral nervous system.

Doe *et al.*^{1,2} have recently tested the role of the *Drosophila* genes *even-skipped* (*eve*) and *fushi tarazu* (*ftz*) in the differentiation of two sibling pairs of identified neurons: *eve* and *ftz* are 'segmentation' genes, whose function during early development is to partition the blastoderm into equipotential germ-band segments (segmental identities are largely controlled by

switching' experiments that have been done on cellular equivalence groups in nematodes, leeches and grasshoppers⁶. In insects, the last mitosis in a neuronal lineage generates two cells which are initially equivalent, although the two sibs will usually differentiate into distinct neurons. Killing one of the cells soon after birth in every case forces the survivor to differentiate into the same 'dominant' one of the two possible outcomes. In the case of *eve* inactivation, it seems that one of the two possible outcomes is also dominant (such as RP1), although here both cells survive.

Transformation between neurons can also happen outside the central nervous system. In a recent issue of *Nature*, Blochlinger *et al.*³ examine the *Drosophila* gene *cut*, a complex genetic locus where some alleles transform surface sensory structures into internal ones. The insect epidermis is decorated with a stereotyped array of sensilla, which usually consist of three structural cells and a single sensory neuron. There are also groups of sensory cells which are found entirely in the interior, such as stretch receptors.

By sequencing the region of the *cut* locus where loss of function transforms

sensory cells, Blochlinger *et al.* find an open reading frame encoding a large protein of nearly 2,200 amino-acid residues that also includes a homoeo domain. By generating an antiserum to part of the predicted sequence, these authors find that the protein is expressed in external sensillar nuclei but not in any of the internal sensory cells. The timing and location of *cut* protein suggest that it regulates other genes necessary for normal development of external sensory structures.

Insect sensory structures arise from epidermal mother cells by simple stereotyped lineages. The switch from a surface to an internal sensory structure could result from transformation of the mother cell itself, generating a new lineage, or through transformations of each of the progeny, switching them independently. Additional lineage studies could be valuable in resolving when and where the *cut* action occurs.

On page 151 of this issue Saint *et al.*⁴ report the cloning and sequencing of the gene *rough*, another *Drosophila* homoeobox-containing gene essential for sensory cell development. The *rough* mutants typically have defective compound-eye structure. Unlike other *Drosophila* homoeobox-containing genes, *rough* is not expressed during embryogenesis. The product appears during the third larval stage, when the photoreceptors become specified through a series of intercellular contacts along the advancing morphogenetic furrow of the eye disk. Saint *et al.* find *rough* messenger RNA along and behind the disk furrow as well as in the larval brain. Loss of *rough* function usually results in ommatidia with reduced numbers of photoreceptors. To pinpoint the role of *rough* function, a developmental analysis of mutant eye disks will be needed where the specific cell contacts can be resolved by electron microscopy.

A final example of a homoeobox-containing gene that is required for normal sensory neuron specification appears in a recent issue of *Cell*. In that study, Way and Chalfie⁵ characterize the nematode gene *mec-3*, whose normal function is essential for the differentiation of a class of six touch-sensitive neurons. Although about 20 genes have been identified that in various ways alter the normal function of the touch cells, the *mec-3* gene alone seems involved in specifying the neurons without altering the cell lineage. In loss-of-function *mec-3* mutants, each touch cell

develops into an as-yet unidentified neuron. Furthermore normal *mec-3* function is necessary for a second mutation, *mec-4(d)*, to cause touch-cell degeneration. An intriguing possibility is that the regulatory DNA of the *mec-4(d)* gene is bound by the *mec-3* protein.

Do these examples demonstrate a general role for genes with homoeo boxes that will apply to all neurons? The answer to this question is particularly significant for understanding vertebrate genes with homoeo boxes, because many of these are expressed in the central nervous system⁶. Unfortunately, the evidence demonstrat-

ing a role for the homoeo box in the nervous system is still slim, because it is difficult to examine the development of singly identified neurons, and because there are only a few systems where both detailed genetics and developmental analyses are feasible. By combining these approaches with molecular methods, as has been done with nematodes and fruit-flies, it is now possible to probe deeply into the role of specific kinds of genes in the development of the nervous system. □

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Ruffled feathers calmed by fossil bird

NEW specimens of *Archaeopteryx lithographica*, the earliest known bird, are not found every day — in fact until now only five have been identified. This makes the recent announcement of a sixth especially exciting (Wellnhofer, P. *Science* 240, 1790; 1988). This individual had previously been assigned to the chicken-sized thero-

printing industry for its evenness and quality in lithography, hence the specific name of the fossil. The new specimen was collected by the former mayor of Solnhofen, who did not realise the importance of his find until it was identified as *Archaeopteryx* by staff at the Eichstatt museum. The subsequent discovery of feather shaft impressions precludes any possibility that these may have been forged. In 1986, astronomers Hoyle and Wickramasinghe claimed that delicate feather prints in other *Archaeopteryx* specimens had been added to theropod skeletons in an elaborate museum hoax, charges strongly denied by the British Museum (Natural History). The uncharacteristically strong wording of the title of the Museum's rebuttal — "*Archaeopteryx* is not a forgery" — shows how high feelings were running at the time (Charig, A. J. *et al. Science* 232, 622; 1986).

The debate derives its intensity from the phylogenetic significance of the possession of feathers: the only character that definitively separates birds from all other groups. Because *Archaeopteryx* is generally considered a close relative of theropods but is relatively primitive in many features other than the possession of feathers, some researchers have suggested that feathers are a shared, derived character of birds and certain theropods. One restoration of the dinosaur *Syntarsus*, for example, gives it a feathery crest. But such ideas remain fanciful, as there is as yet no hard evidence to support the presence of feathers in any accredited dinosaur group. Others consider the relatively late appearance of birds in the fossil record anomalous, favouring a much earlier appearance and a relationship with early crocodiles rather than dinosaurs. Triassic fossils from Texas (Beardsley, T. *Nature* 322, 677; 1986) may lend credence to this view but their identification as primitive birds has not yet met with general acceptance. Until then, playful suggestions that fossils of *Compsognathus* can be forged by removing feather impressions from *Archaeopteryx* specimens are not regarded as plausible.

Henry Gee

Henry Gee is on the editorial staff of Nature.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Bone of contention — *Archaeopteryx*, courtesy of the British Museum (Natural History).

pod dinosaur *Compsognathus* but now detailed preparation of the fossil has shown up impressions of the shafts of feathers.

The specimen, complete except for most of the skull, is by far the largest yet found, some 10 per cent larger than the specimen in the British Museum (Natural History) in London (see figure) and fully twice as big as that in the museum in Eichstatt, West Germany. This will lend weight to the contention that two species are represented in the collection, the five largest being *A. lithographica* while the Eichstatt specimen is removed to *Archaeopteryx* sp. or even a new genus, *Jurapteryx recurva*.

All *Archaeopteryx* specimens come from the extremely finely grained limestone of Solnhofen in Bavaria, renowned in the

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4. Saint, R., Kalionis, B., Lockett, T.J. & Elizur, A. *Nature* 334, 151-154 (1988).
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Oceanography

The potential of vorticity

Thomas Keffer

VORTICITY is a central feature of theories of ocean dynamics, being used to simplify the complex equations of global fluid dynamics. The natural 'planetary vorticity' of the Earth's rotation dominates the equation describing the conservation of vorticity, and perturbations about this leading term are numerically easy to handle in calculations of the ocean's behaviour. Now it is recognized that at certain length scales vorticity is intimately linked to the vertical density profile of the ocean. As such, it is an observable feature and can be used as a tracer of ocean movement. L. Talley, in the finest such analysis to date (*J. phys. Oceanog.* **18**, 89–106; 1988), uses vorticity to show that a thick homogeneous layer of cool water in the western subtropical Pacific must have originated during winter storms at the edge of the Pacific basin.

In the autumn of 1740 Captain Henry Ellis, the master of the English slave trader *Earl of Halifax*, lowered a bucket, equipped with one-way valves and a thermometer, into the subtropical Atlantic (*Phil. Trans. R. Soc.* **47**, 211–214; 1741). He noted that the "cold increased regularly with, in proportion to the depths . . . to 3,900 feet, from whence the mercury in the thermometer came up at 53 degrees [12°C], and tho' I afterwards sunk it to the depth of 5,346 feet . . . it came up no lower." It was the first recorded measurement of the thermocline in the ocean. Although Ellis's temperatures were too high, perhaps because of leaky valves and conduction through the wooden bucket, he had the essential shape of the subtropical temperature profile right — a strong gradient near the surface, less so with depth.

Explaining this obvious and fundamental feature of the ocean has turned out to be surprisingly difficult. Although it was quickly recognized that the cold water at abyssal depths (below 2,000 metres) could come only from polar regions; it took much longer to recognize that the shape of the thermocline itself comes not from vertical mixing with the warm surface waters, but from slanting, sinking motions within the subtropics itself (Iselin, C. O'D. *Trans. am. geophys. Un.* **20**, 414–417; 1939). This is confirmed by the near similarity of a vertical temperature profile at a location to a horizontal profile taken to the north, over a scale of perhaps 1,000 km.

Recently, much progress has been made on the theoretical side of the problem (see, for example, Holland, W. R., Keffer, T. & Rhines, P. B. *Nature* **308**,

698–705; 1984). This has come from the recognition that the vertical profile of temperature (or alternatively, the distance between isotherms) is intimately linked with the vorticity field. Just as a figure skater can change his angular velocity by pulling his arms in and decreasing his moment of inertia, a fluid parcel can change its angular velocity by increasing the distance h between isotherms. Yet, in some sense, both the skater and the fluid parcel conserve their total vorticity. This



Perhaps the most striking difference of the North Pacific from the North Atlantic is in the deeper layers. In the North Pacific these layers do not outcrop with the sea surface. Isolated from surface forcing, fluid parcels are free to make many trips round the gyres, sharing their properties with other parcels. The result is that they arrive at a nearly uniform value of properties within the gyre. The figure shows potential vorticity in a density layer chosen to show the onset of this homogenization (density between 1.02625 and $1.02675 \text{ g cm}^{-3}$). The subpolar gyre outcrop appears only in its northwest corner and elsewhere shows signs of vertical mixing downward from the fresh halocline above. In the more dynamically isolated subtropical gyre, however, homogenization is nearly complete. A plume of high potential vorticity enters from the subpolar gyre on the eastern side, illustrating the tracerlike characteristics of this dynamical variable.

conserved vorticity is termed the potential vorticity. In the case of a fluid parcel, the relevant angular velocity (at these large scales) is not the spin seen by an observer on Earth (as with the figure skater), but the component of vorticity imparted by the rotating Earth that is parallel to the Earth's axis. This is given by the Coriolis force f , which is a simple function of latitude: $f = 2\Omega \sin(\text{latitude})$, where Ω is the angular velocity of the Earth's rotation. The total potential vorticity of a fluid layer is then simply f/h .

Just as the skater can partition his potential vorticity as he wishes between angular velocity and moment of inertia by pulling his arms in and out, the fluid parcel can partition its potential vorticity between latitude and thickness. Herein lies the usefulness of potential vorticity: because it is conserved, if you know the initial potential vorticity when a fluid parcel leaves the surface, then you can predict h , the distance between isotherms, everywhere along the parcel's path, as it

changes latitude. If this path can be predicted (through the use of additional dynamical constraints) then the structure of the thermocline can be predicted everywhere in the gyre.

L. Talley's analysis of the potential-vorticity field of the North Pacific is a fine example of this approach. This ocean makes an interesting comparison with the North Atlantic because only its shallowest and lightest density surfaces intersect (outcrop) with the sea surface, and the heaviest surfaces are unventilated. In contrast, the North Atlantic is extensively ventilated and even the heaviest surfaces outcrop occasionally. Not surprisingly, it is the light surfaces of the two oceans that are the most similar. For example, Talley notes the existence of a Pacific variety of

'subtropical mode water', a variant of the North Atlantic's 18° water. A mode water is a thick thermostat of water, all at the same temperature. Because its distinguishing characteristic is vertical homogeneity (that is, a large h), it shows up as an easily tracked potential-vorticity minimum (small f/h). Subtropical mode waters, both Atlantic and Pacific varieties, are remnant deep-winter mixed layers formed on the western edges of their respective oceans by the intense cooling and evaporation caused by storms from the nearby continents. The resulting deep mixed layers can be subsequently capped off, subducted and appear remotely from their site of formation, detectable as a minimum in potential vorticity. Talley notes that the signatures of the two varieties are similar and detectable far from their formation sites.

At slightly higher densities, the oceans' characteristics begin to differ. Both oceans have a variety of subpolar mode water which, like subtropical mode water,

is a thermostad, but one formed in sub-polar gyres. Talley shows how the two circulation patterns are strikingly similar, but that the North Pacific's version occupies a narrower depth band. This is because the strong, shallow halocline (salinity gradient) in the North Pacific which limits mixed-layer depth to perhaps 200 m, as against 1,500m in the North Atlantic.

On the deepest and least ventilated layers of the North Pacific, the differences from the North Atlantic are the most profound. Free from external sources of potential vorticity, these layers show nearly complete homogenization of potential vorticity (see figure).

Talley notes a general correlation between 'Ekman' upwelling (the divergence of fluid in the directly forced surface layers of the ocean), and high near-surface potential vorticity. Although there are three different regimes showing this corre-

lation (the tropics along the eastern boundary and north of the intertropical convergence zone; the eastern subtropics; and the eastern subpolar gyre), the causal link is not clear. Ekman upwelling can be expected to cause a doming of density surfaces within the gyre, but an upwelling that decays with depth would actually cause a thickening of the near-surface layers and a minimum in potential vorticity. A possible causal mechanism for these maxima is the downward vertical mixing of strong seasonal stratification caused by the heavy rainfall in these regions. The observed correlation would then be an indirect one, caused by the correlation of cyclonic winds with both heavy rainfall and Ekman upwelling. □

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Neuroethology

Birdsong and neurogenesis

Peter Marler

THE discovery that new neurons arise in the brain of adult canaries¹ has heightened the already considerable interest of neurobiologists in mechanisms of song learning in birds. A new study reported by Nordeen and Nordeen on page 149 of this issue² increases the probability of specific links between neurogenesis and song learning by demonstrating a close temporal correlation between a surge of new neuron recruitment in the brains of young zebra-finch males, and processes of acquiring and developing song.

Unlike many birds, in which song acquisition and development are restricted to a short period in adolescence, a male canary retains song plasticity throughout the first few years of life; other, related, finches can learn new calls as long as they live. This extended behavioural flexibility implies some form of continued relearning both by males and probably also by their mates, who have to respond to the new calls. Because a significant number of the new neurons end up in one of the brain nuclei known to be involved in song control, it seemed reasonable to link neurogenesis to vocal learning. But the precise nature of the relationship in the canary has remained elusive.

The new work² of Nordeen and Nordeen helps to elucidate this problem. In zebra finches, song development is compressed into a short period early in life. Rates of incorporation of new neurons at this time are far greater than after the process of song development is completed in adulthood, and greater than in the brains of young females, which do not sing. Moreover, Nordeen and Nordeen

discovered that a large proportion of the new neurons in hyperstriatum ventralis pars caudalis (HVC), the highest centre for the integration of auditory and motor information in song control, extend neuritic processes to the nucleus robustus archistriatalis (RA), generally thought to be the main centre for motor organization in the forebrain. Thus in young male zebra finches, neurogenesis affects at least two links in the chain of command for song production. If methods to modulate neurogenesis can be developed, manipulation of the mechanisms by which new neurons are generated should reveal whether the predicted impact on song development occurs. Such studies will eliminate any lingering uncertainty about whether neurogenesis actively sustains learning or is in some way a consequence of neural participation in the process of song acquisition and motor development. Whatever the precise nature of the causal link, the study by Nordeen and Nordeen increases the likelihood of a causal relationship between neurogenesis and song learning.

Many other issues remain to be explored. The rates of neurogenesis found in the brains of young female zebra finches, which do not sing, are by no means insignificant, as is true of adult male zebra finches, even long after closure of the sensitive period for song learning³. As mentioned above, neurogenesis was first discovered in female canaries, which do not normally sing either. Subsequent research revealed that new neurons are produced in adulthood, not only in songbirds but also in bird species that display no evidence of vocal learning, such as ring

doves³. What functions do the new neurons serve in these cases?

Nottebohm suggested that adult neurogenesis in birds is related to the updating of perceptual memories in neural systems that have limited space for learning. This speculation derives from another finding, namely that individual and species differences in the volume of some song-control brain nuclei are correlated with the size of the vocal repertoire^{4,5}. Thus, Nottebohm hypothesizes⁶ that "the number and complexity of brain circuits available for a particular skill may limit how much can be learned". If birds must constantly update perceptual representations of the sounds of mates and other companions, the limitations on brain space may require the neuronal turnover provided by neurogenesis, occurring on a scale that probably replaces a large proportion of neurons in HVC each year.

Note, however, that the new neurons are found throughout the forebrain. Although no quantitative documentation of their distribution is yet available, it appears from published figures³ that the concentration of new neurons, although low in RA, is as high in other regions of the forebrain as it is in HVC. It is not yet known whether the distribution of the sites to which new neurons migrate from their place of origin (the walls of the lateral ventricles of the forebrain) coincides with the predicted involvement in renewed perceptual processing and representation, either of vocal signals or of other stimuli.

When first described, the claim of adult neurogenesis in the bird brain was greeted with scepticism. Such neurogenesis is known to occur in fish and other cold-blooded vertebrates⁷, perhaps associated with problems of continued growth throughout life, such as change in eye size. By contrast, birds reach adult size at an early age, so avian adult neurogenesis may be different from that of other vertebrates. In mammals, formation of new neurons is largely restricted to early stages of ontogeny, apart from special cases such



100 years ago

FROM a report signed by Mr. Edward S. Holden we learn that the trustees of the Lick Observatory . . . have provided a photographic attachment to the 36-inch telescope, which will enable this to be used as a gigantic camera. In the photography of the moon, of the planets, of nebulae, and comets the Lick telescope will have some important advantages. "But," says Mr. Holden, "it is in the photography of stars — of double and binary stars, of all the fainter stars — that the Lick telescope will find its chief application and demonstrate its immense superiority.

From *Nature* 38, 257; 12 July 1888.

as the hippocampus and the olfactory bulb, some of which are still controversial.

Hitherto, birds were assumed to be like mammals, which is why Nottebohm and colleagues went to unusual lengths to render their findings generally acceptable. They showed that new neurons are recipients of synapses⁸, but glial cells sometimes receive such synapses. Concerns about guidance and rates of migration of new neurons were allayed by the discovery of radial glia in adult canary brains⁹. Present in adult fish and amphibia, but only in developing mammalian brains, these are thought to provide supports for directed neural migration.

In a technical *tour-de-force*, the functional involvement of new neurons into existing circuits within HVC was demonstrated by impaling cells with electrodes, recording their responses to sound, marking them and identifying them post-mortem as having incorporated radioactive thymidine¹⁰. Interestingly, most of the new neurons in HVC appear to have the status of interneurons in the canary, only a few projecting to other song-control nuclei¹¹. This is another contrast with the results of Nordeen and Nordeen in this

issue², who find that more than half of the new neurons in the young male zebra-finch brain project to the RA, and so potentially engage the motor circuitry for song control. This suggests a different emphasis in the functions assumed by new neurons in brains of young birds and adults. Moreover, aside from the potential for damage repair¹², the function of adult neurogenesis in the bird brain continues to remain a mystery and a challenge. □

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Radioastronomy

Smaller is more beautiful

Peter Scheuer

IN the early 1950s, when radioastronomers were struggling to get a primary beam narrower than 1°, who would have expected to see a radio map, such as that on page 132 of this issue¹ obtained by Bartel *et al.*, with an angular resolution 10,000 times better than that of optical telescopes?

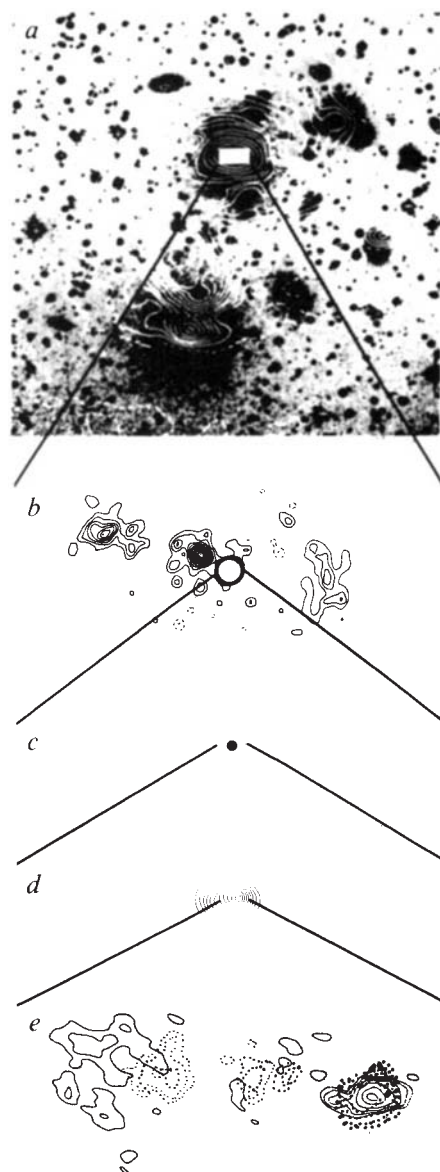
The key to better angular resolution is interferometry: using arrays of separate but linked radio telescopes to simulate one large aperture. Around 1960 Martin Ryle introduced the modern form of aperture synthesis, exploiting the Earth's rotation to move the telescopes. Then better receivers allowed shorter wavelengths to be used. By the mid-1970s, these advances together gave the Cambridge 5-kilometre telescope a resolution of 0.7 arcseconds, as good as optical telescopes at the best sites. Less complete information on structures 10 times smaller was obtained too, notably by Henry Palmer and co-workers at Jodrell Bank, using interferometers with baselines of the order of 100 kilometres joined by radio links. Their instrument was the forerunner of the MERLIN array centred at Jodrell Bank.

The development of videotape made it possible to record the whole intermediate-frequency signal from each radio telescope, together with 'time-pips' from a

maser clock. This dispensed with the need for hardware connections between the telescopes of an interferometer array. The signals on pairs of tapes are multiplied centrally to give the 'interferometer outputs'. With that freedom, interferometers could extend across oceans, and VLBI (very-long-baseline interferometry) was born.

The basic technical problem with VLBI is that phase errors of the individual telescopes cannot be determined absolutely or even kept constant: even if the geometry of the system were known to, say, the nearest millimetre, and times measured to a light-millimetre, there would still be a column of atmosphere above each telescope introducing an unpredictably varying electrical path length.

The consequent lack of information about fringe phases, familiar to X-ray crystallographers, can be partially overcome by using 'closure phase'. Consider three telescopes A, B and C, in an observing run. Adding up the observed fringe phases for baselines AB, BC and CA cancels the phase errors for the individual telescopes, so this closure phase gives genuine, but complicated, information about the structure of the radio source. The principle had been known for a long time, but Readhead and Wilkinson² brought it out of obscurity and made it a



Approaching the active nucleus of NGC1275: the scale increases by a factor of 15 between successive maps. The first³ (a, 810 arcseconds across) shows the 10-minute halo, observed with the Westerbork Synthesis Radio Telescope, superposed on an optical photograph; b, a MERLIN map⁴; c, on this scale the source is a point; d, VLBI map⁵ at 50 centimetres; e, VLBI maps⁶ at 1.3 centimetres in April 1981 (dotted contours) and February 1985. Because VLBI does not give absolute positions, superposition of the two maps in e represents a guess that the northern component is the 'core'. The new map on page 362 of this issue¹ shows further detail within that northern component. (Maps reproduced by permission of A. G. de Bruyn, A. Pedlar, P. N. Wilkinson, the Royal Astronomical Society and Cambridge University Press.)

practical tool for VLBI. For an array of N telescopes there are $\frac{1}{2}N(N-1)$ baselines but only $N-1$ unknown phase errors, so most of the phase information is recoverable when many telescopes take part in one experiment.

But first there must be some observable fringes. Linear phase drifts are dealt with by searching each baseline (each pair of

tapes) for the delay and fringe rate that gives the highest correlation between the signals. But typically one can integrate the signal coherently for only 10 minutes, compared with 12 hours or more for conventional instruments. Thus, even experiments using several of the largest radio telescopes cannot approach the sensitivity of the Very Large Array in New Mexico.

The dynamic range of VLBI maps has been improved greatly as more radio observatories have acquired VLBI equipment, improving the sampling of the Fourier transform of the radio map. Up to 18 independent groups now routinely record compatible data simultaneously. Often experiments involve telescopes in Europe and North America and occasionally telescopes in countries whose governments are not on speaking terms.

The resolution limit is set by the number of wavelengths that fit on the Earth's diameter. Unfortunately the technical problems worsen rapidly as one tries to work at shorter wavelengths. Nevertheless, VLBI fringes have been observed successfully at wavelengths as short as 3.4 millimetres, and the paper by Bartel *et al.*¹ in this issue from eight participating observatories, reporting the production of a real map of a radio source at 7-millimetre wavelength with a resolution of 10^{-4} arcseconds, is something of a landmark. The source is NGC1275, an unusual radio galaxy whose active nucleus is among the brightest milliarcsecond structures in the sky at 7 millimetres. It is relatively near (redshift 0.018) so that the linear resolution is 0.05 parsec. That is still very large compared with the galaxy's central engine according to black-hole-based models (the Schwarzschild radius for a 10^8 solar-mass black hole is 10^{-5} parsec), but the radius of the surrounding accretion disk is quite uncertain and there might be some direct evidence for the disk at this resolution.

The original drive for VLBI came from the strange behaviour of some quasars and radio galaxies whose flux density varied faster than expected, suggesting relativistic expansion. The 'superluminal motion' that was discovered was even stranger: it seemed that a lumpy jet was escaping from the (presumed) nucleus at 5–15 times the speed of light. Many explanations have been proposed, including relativistic beaming, gravitational lensing, and non-cosmological redshifts for quasars. Further complexities have become apparent, including bent jets and jets with knots that are stationary (or moving backwards, depending on one's interpretation).

NGC1275 is itself unusual in that it expands, but with an apparent speed less than the speed of light; if its nucleus ejects highly relativistic jets as postulated in the standard model, they are either directed at a ridiculously small angle to the line of sight or (more plausibly) we are watching

some feature such as a shock front that does not move as fast as the matter.

Many other uses have been found for VLBI. Geodetic uses are described by Thomas A. Herring on page 102 of this issue². Within our Galaxy, VLBI can distinguish the OH and H₂O maser spots in nebulae and near the surfaces of giant stars and measure their relative motions; there are very luminous 'megamasers' that can be measured even in other galaxies. At the longer wavelengths, the small-scale structure of the interstellar medium of our Galaxy is revealed in distorted VLBI images of small sources beyond. The interstellar medium of remote galaxies that lie on the line of sight to distant quasars can be studied by hydrogen absorption at 21 centimetres. And efforts are being made to measure the expansion of recent supernovae in nearby galaxies (which, together with emission-line profiles, could give new distance estimates) and to detect the proper motions of the nuclei of nearby galaxies.

Surprisingly, VLBI observations are limited less by telescope time than by the supply of Mark-III system tapes; the most important advance now in progress is the 12-fold increase in track density on the tape-recording heads. In the longer term, a dedicated VLBI array, of ten 25-metre telescopes in North America, usable up to 43 gigahertz, should come into full operation in 1993. Still better resolution will be achieved by reducing the wavelength (as more millimetre-wave telescopes acquire VLBI terminals) and probably also by increasing the physical baseline. Fringes from an Earth-to-satellite interferometer have already been demonstrated³. In Europe, QUASAT will be in competition with other projects for approval by the European Space Agency in October this year. This would put a 15-metre telescope operating at frequencies up to 22 gigahertz into an orbit extending to 8 Earth radii. Radioastron (Soviet Union) and VSOP (Japan) are other space VLBI projects in an advanced planning stage.

Finally, the new ideas about interferometry are being returned to their original domain as radioastronomers take up VLBI at micrometre and submicrometre wavelengths. Seventy years on, the Michelson stellar interferometer is coming back to the optical band. □

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Daedalus

Perfected markets

THE stock-market crash of 1987 has been blamed on the deficiencies of current computer-trading programs. So Daedalus is improving them by strict darwinian methods. He is setting up a big computer model of the stock market: a simulated arena in which rival trading programs can compete. Some will rapidly go bankrupt; Daedalus will mutate the survivors in search of still greater efficiency, and ultimately an optimum program will emerge. The whole process mimics the emergence of the 'evolutionarily stable strategy', or ESS, that governs competition between animals of the same species. Once an ESS is widespread in a population, no mutant alternative can ever defeat it. Similarly, the winner of Daedalus's computer contest should be a quite unbeatable financial ESS, or FESS. DREADCO will then sell copies of FESS, at a suitably vast price, in the financial community at large.

Like the ESS's of the animal kingdom, FESS will have a cautious, shifty, opportunistic character. Always alert to seize small, safe gains, it will rapidly shuffle away from major threat or challenge, avoiding serious gambles in favour of living to fight another day. Faced with FESS, more defensive programs will slowly bleed bankrupt by small losses; more heroic risk-takers may soar triumphantly above it for a while, but will ultimately crash just as spectacularly.

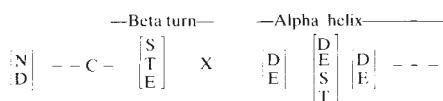
Once it becomes clear that nothing can beat FESS, all institutions will adopt it. FESS computers will take over the market, and the whole parasitic tribe of market dealers will have to find more productive employment. But FESS will not stabilize the market; indeed, it will probably gyrate more wildly than ever. A perfect market is based on pure fashion: no program has any basis for the value it assigns a share or currency, other than the equally baseless opinions of identical competing programs. Such a totally introverted system could drift indifferently between stock market indices of one, or a thousand, or a million. Some stability may be introduced by objective information (like dividends of firms whose shares are being traded), which would have to be transmitted to all FESS players simultaneously, to avoid bias or insider dealing.

A fully FESS-computerized stock market will lose its hypnotic importance. But all FESS players will still make steady money, from an inevitable fringe of mavericks trying to beat the system: in just the way that the bookmaking community makes steady money from those romantics who, irrationally entranced by long odds, persist in placing their doomed money on hopeless outsiders. David Jones

Cell-division sequence motif

SIR—We wish to point out that a structural motif shared among three transforming protein classes, the papovaviral large-T antigens, the adenoviral E1A proteins and the v- and c-myc oncoproteins^{1,2} is also present in the E7 transforming proteins of human papillomaviruses³ and the CDC25 gene, that is a mitotic regulator of yeast. The region encoding the shared motif has recently been demonstrated to be required by the large-T antigen of simian virus 40 (SV40) both for transforming function and for its specific binding to the 'negative oncoprotein' RB⁴, the putative human retinoblastoma gene product, to which the E1A protein also binds specifically⁵. In addition, this region of SV40 large-T, E1A and human c-myc has been shown to be required for co-transformation with *ras* oncogenes of primary cells (see bibliography in ref. 1).

The motif is:



where the brackets indicate alternatives at a given position; the dashes, any amino acid; N, asparagine; D, aspartic acid; C, cysteine; E, glutamic acid; S, serine; T, threonine; and X, a spacer of 1–8 amino acids, often containing a proline and serines. There is a strong correlation between the presence of an aspartic acid in the first position and a hydrophobic amino acid in the second position. The predicted alpha helix is dominated over the remainder of its length by polar residues. Studies on the SV40 large-T and the adenoviral E1A proteins show that mutation of the glutamic acid in the sixth position of this descriptor to a positive amino acid inactivates the transforming activity of the proteins¹. In one of the v-myc oncoproteins, at least one serine/threonine site in this region is phosphorylated⁶. This may also be how the region is activated in normal cellular proteins that contain the motif, while in some oncoproteins activation may be the result of the substitution of serine or threonine by glutamic acid or aspartic acid. This fits the apparent and unusual equivalence of all four amino acids in at least one position in the motif.

Using a sequence regular-expression handler and a modified Chou–Fasman secondary-structure prediction algorithm to annotate sequences with potential secondary structure, all matches to the descriptor within the NBRF protein sequence database (release 16) and the translated Genbank database (release 55) were identified out of over 11,000 proteins. They included nearly all of the v-myc and c-myc proteins, nearly all of the E1A and large T proteins, and six of the eight E7

proteins. Additional matches among proteins related to either cell transformation or division include a deer papillomavirus protein, a glycoprotein of Rift Valley fever virus and, perhaps most importantly, the CDC25 protein of yeast⁷. Only 12 functionally unrelated proteins were clearly identified. It is difficult to assess whether or not the pattern fails to match other known transforming proteins (for example, fos, jun and myb) because their molecular mechanisms are different or because the pattern itself is incomplete. Given the functional complexity of oncogenesis, we currently favour the former and therefore, believe the pattern's specificity to be nearly 100%. In which case, this may be one of the most diagnostic protein functional patterns known.

The importance of the match with the CDC25 protein is that it interacts with a known protein kinase WEE1+ and apparently modulates the activity of CDC2, another mitotic regulator protein⁸; interestingly, the ability of CDC25 to initiate cell division shows dosage effects, which are compatible with a protein–protein binding titration, such as is now known for the interaction of SV40 large-T or E1A with RB.

Thus, we propose that the majority of proteins containing the motif carry out one of the required steps in the cell division competency cascade of deactivating a cell division repressor (or activating a cell division activator). That these proteins will only transform primary cells in the presence of *ras* oncogenes suggests that at least one other cell-division signal transduction pathway exists.

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Reversal of acidification

SIR—Battarbee *et al.*¹ recently provided a reassuring account, based on diatom and lake-water records, that lake acidification in Scotland has either been reduced or has been reversed because of reductions in SO₂ emissions since 1970. Our recent modelling results simulating long-term changes in the acidity of Scottish lake and stream waters^{2,3} also suggest a recent

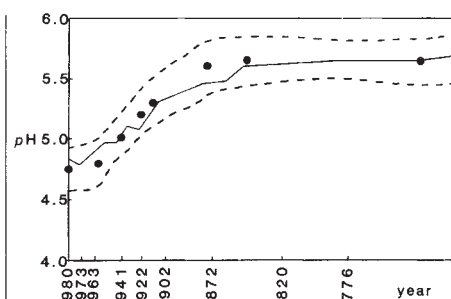


Fig. 1 Round Loch of Glenhead simulation of pH compared with palaeoecological data¹. Solid line, palaeoecological reconstruction; dashed line, MAGIC reconstruction with 95% confidence bounds; dot, MAGIC reconstruction.

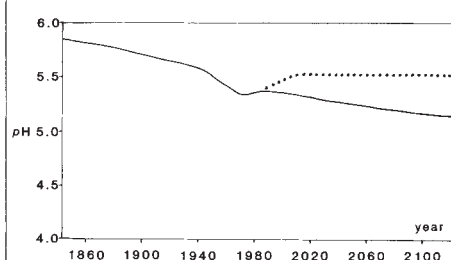


Fig. 2 Dargall Lane stream pH MAGIC simulation². Solid line, prediction with future emissions at 1985 values; dotted line, prediction with emissions declining a further 50% from 1985 values over the next 20 years.

stabilization for some catchments.

Figure 1, for example, shows a pH simulation for Round Loch of Glenhead (south-west Scotland), using the MAGIC model, which compares well with the diatom reconstructed pH¹. Figure 2 shows the pH response for Dargall Lane, a stream draining into Loch Dee in the Galloway region of south-west Scotland, which shows similar declines over the past 100 years up to the 1970s. The model response also indicates a recovery in the 1980s which corresponds closely with the recovery of Battarbee *et al.*

Long-term predictions with the MAGIC model suggest that the recovery or stabilization is probably temporary, provided recent (1985) deposition levels are maintained. Qualitatively, this result may seem surprising. But the predicted decline results from the low rate of weathering and the highly base-depleted cation-exchange store which continues to be depleted at current acidic-oxide deposition rates. To avoid further declines in stream pH and ensure a significant recovery, for Dargall Lane, larger reductions in deposition of acidic oxides are required. To stabilize stream pH to provide a very modest improvement at Dargall Lane, for example, a further 50 per cent reduction in deposition from 1985 levels is needed, according to the estimates in Fig. 2.

Different catchments will show differ-

ent responses dependent on factors such as soil-base saturation levels, sulphate adsorption and release mechanisms, weathering rates, hydrological factors and deposition rates. Although there are many uncertainties in the MAGIC model⁷, the diatom records suggest that the model is applicable under these circumstances. Indeed, the model provides the only means of making site-specific or regional predictions of long-term future behaviour of stream and lake acidity.

On this basis, the observed improvements in Scotland, which are also suggested by the MAGIC model, should not be viewed as a case against further controls of acidic oxide emissions. Indeed, the modelling evidence suggests that further large-scale reductions are required and the amelioratory effects will be relatively rapid. Preliminary estimates for the United Kingdom, using the MAGIC model, indicate deposition reductions of around 50 per cent from 1985 levels are required to obtain significant long-term recovery¹⁻⁸ for much of the British uplands.

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Punctuation and selection

SIR—Radical theoretical positions in science are like programme trading in the securities markets: during euphoric uptrends their authors are not averse to taking full credit; but in the downward spiral that inevitably follows when reason returns, those associated with the movement no longer wish to be acknowledged.

This parallel came to mind as I read Steven Stanley's charge¹ that John Maynard Smith, in his criticism² of Eldredge and Gould's punctuational model of evolution, has seriously misrepresented Stanley's writings on species selection. Actually, Maynard Smith mentioned Stanley only twice. The second mentioned deals with a dispute over whether the evidence for punctuation has been in the form of measured morphological change or, instead, the duration of named taxa in the fossil record. Stanley does not mention this in his reply, so we can assume that his discomfort focuses on the other point: the decoupling of macro-

evolution from microevolution, and the role of random elements therein.

Specifically, Maynard Smith is charged with having "quoted out of context" a statement of Stanley's that refers to a "strong random element" in speciation. I have checked the passage in question (p.187 of ref. 3) and find my reading to be the same as Maynard Smith's. Indeed, shortly afterwards (p.193 of ref. 3) Stanley makes the case that species selection should not be considered as a subdivision of natural selection, and he asserts (p. 212) that "transitions are opportunistic in nature, reflecting the 'experimental' nature of speciation".

In the matter of quotes out of context, however, readers should consider Stanley's own statement that "although he never considered the process in detail, Wright asserted that selection operates at the level of the species...." Wright's paper¹ thus referenced is his classic *Modes of Selection*, in which the concluding sentence takes the rather more eclectic stance that "The course of evolution of vertebrate life and of life in general has been guided throughout by a hierarchy of processes of selection ranging from selection between genes to selection between orders, classes and even phyla." Significantly, the same paper opens with what Wright called the dogmatic statement of his general position with regard to evolution: "Adaptation rather than mere change seems to me to be the central problem. The only mechanism for evolutionary adaptation that has held up under investigation is natural selection." In Stanley's main publication on patterns of evolution³, the concept of adaptation is conspicuous by its virtual absence.

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Understanding aggression

SIR—Had Melvin Konner been willing to take our book (*Aggression: Conflict in Animals and Humans Reconsidered*) a little more seriously, he might have understood it rather better¹. We are not, as he suggests, anti-sociobiological; on the contrary, we endorse the sociobiological enterprise and attempt to reassess the significance of evolutionary theory for an understanding of aggression.

In this process of reassessment we do indeed criticize the work of several sociobiologists, including Konner himself (though not, as he suggests, either Robert Axelrod or Richard Dawkins). Our quarrel with Konner has to do with his

use of selected ethnographic material to support the view that "no cultural training, however designed, can eliminate the basic core of capability of violence that is part of the makeup of human beings"². Since our book was sent to press, the authors of the relevant ethnographic material have themselves complained about this misuse of their work³.

The proper response to criticism is counter-criticism. Konner ignores this and all other substantive issues, contenting himself instead with a rhetorical plea: "Respected critics of sociobiology! Surely you can do better than this!". The answer is obvious: "Respected sociobiologists! Surely you can do better than Konner!".

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Segregation of cystic fibrosis allele

SIR—Kitzis *et al.* (*Nature* **333**, 215; 1988) suggest that the high frequency of the cystic fibrosis (CF) mutant gene in Caucasian populations may have arisen from the preferential inheritance of the CF chromosome from male to male. This intriguing hypothesis was based on the haplotyping of 60 asymptomatic siblings of CF patients by DNA probes. Twenty of 22 normal homozygotes were girls, 16 of 21 paternal CF chromosomes were inherited by boys. This is at odds with our own haplotyping of 60 unaffected siblings from 41 German CF families. Ten girls and 10 boys were typed homozygous normal. Eight of 17 paternal CF chromosomes and 15 of 23 (65%) maternal CF chromosomes had been passed to boys.

If one compiles the data from the two studies, the proportion of maternal and paternal CF alleles is about 1 to 1 in both male and female CF carriers, as expected for autosomal mendelian inheritance. Hence, an unfortunate sampling bias may be the most likely explanation for the unexpected segregation of the CF allele to the male germline observed by Kitzis *et al.* The data still do not exclude the possibility of a slightly more frequent transmission of CF chromosomes to males. More extensive data from pedigree analyses and population studies on unrelated individuals should settle the issue.

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Jeux sans frontières

Sheldon Krinsky

The Biotechnology Revolution: An International Perspective. By Alan M. Russell. *Wheatshaf, Brighton, UK/St Martin's Press, New York: 1988. Pp. 266. £35, \$45.*

WHEN gene splicing emerged as a research tool in molecular genetics, world-wide concern over safety was encapsulated in the apothegm 'microbes do not respect national boundaries'. This phrase carried a meaning to all participants in the complex and sometimes frustrating debates over the governance of recombinant DNA (rDNA) research. Those who advocated *de minimis* voluntary guidelines pointed to the absurdity of some countries imposing containment standards that exceeded an accepted norm. They knew that no country was willing, unilaterally, to forego a strong start in the race to develop biotechnology. The metaphor of organisms with international passports was used to promote regulatory harmonization — a convergence towards uniform minimal safety standards.

Advocates for stringent controls, including those who supported the establishment of several high-containment laboratories in each nation, also exploited the symbol of microbes and boundaries in advancing their case. One such boundary was the putative evolutionary separation between eukaryotic and prokaryotic organisms, so-called species barriers which rDNA technology so easily breached. Species barriers were cited to call attention to the broad evolutionary consequences of the new technology in contrast to narrowly posed risk assessment. The regulatory maximalists also acknowledged the irrelevancy of national borders to peregrine microbes. However, from this insight they concluded that control of this technology should be given over to a world congress or international convention. Both biological and political boundaries played an important symbolic role in the early debates over gene-splicing.

All this is history now. rDNA technology is celebrating its fifteenth anniversary. The science is no longer obscure and practical applications are abundant. There are high-school kits for gene-splicing experiments. Gene sequencing has even been introduced into the courts as a method for identifying criminal suspects. Industrialization of biotechnology has produced new

symbols such as global rDNA markets, patents on life and university-industry partnerships. The international aspects of biotechnology are more complex now than they were in 1975 when scientists throughout the world gathered at Asilomar "to review the progress, opportunities, potential dangers and possible remedies

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Biotechnology makes news — last year the first convictions were made in Britain on genetic evidence.

associated with the construction, and introduction of new recombinant DNA molecules into living cells". Concern about hazards has shifted from the laboratory to the open environment. Today, appeals for regulatory harmonization come most prominently from the multinational companies which proclaim that 'DNA markets have no national boundaries'.

Internationalization of biotechnology means something different to Third World countries, where it raises both hopes and anxieties. The hopes are based on a technology with low capital costs, that is easily transferred, that could be respectful of ecological diversity and therefore efficiently make use of indigenous crops, and that may offer inexpensive vaccines for mass immunization. But the anxieties run deep and for good reasons. Developing countries may face a second generation of

dependency on the industrialized nations through the latter's appropriation of germ plasm and through socio-economic dislocation resulting from substitution of bio-synthetic products for natural ones. The development of replacements for export crops (vanilla from Madagascar, codeine from Turkey, cocoa butter from the Ivory Coast, gum arabic from Nigeria) portends a global agricultural restructuring that some believe is now well underway. Biotechnology is rapidly becoming part of a world economic order.

Alan M. Russell, a lecturer in international relations at North Staffordshire Polytechnic, has produced a book that provides an international perspective on

one of several important stages in the development of biotechnology. It discusses the efforts by nations and international scientific societies to address the safety of rDNA laboratory experiments. The first half of it recapitulates the history of American and British responses to the concerns expressed in the Berg letter, published in *Science* and *Nature* in 1974, and at the Asilomar conference. The author's historical analysis draws exclusively on the existing literature and heavily on the DNA archives at the Massachusetts Institute of Technology. The book adds little to reconceptualizing the historical record, nor does it contribute a new body of critical information. Although generally well researched, the author's account of the development of rDNA guidelines in Britain has a glaring omission in failing to acknowledge *The Politics of Uncertainty*, by D. Bennett, P. Glasner and D.

Travis, a leading scholarly treatment of the subject published in 1986.

In the second half of the book, the author's strengths in international relations, organizational theory and descriptive network analysis are more evident. Russell examines the response by influential international scientific groups such as the European Molecular Biology Organization, the International Council of Scientific Unions and the European Science Foundation to the potential hazards of rDNA research. These bodies served as counter-influences to the tendencies of individual states to set up radically different standards for rDNA research. They set limits on the uncertainty of the risks, consolidated information, sought consensus positions and served as communication channels to strategic policy circles. These organizations operated according to a design: to advance the cause of inter-

national science by counteracting the balkanization of interests.

After the British and American guidelines were published and public debate began, scientists closed ranks with extraordinary speed. In the scientific community there was but a small chorus of dissident voices, primarily in the United States; James Watson referred to these people as "shits, kooks, and incompetents". Russell's conclusion about the consensus within the scientific community holds true for both the national and international groups: "Although it can be questioned whether scientists avoid the pressures of nationalism, especially in large-scale prestige science, it is fair to say that for genetic manipulators the common international fear of excessive regulation enhanced their unity of purpose" (p.209). His analysis is consistent with that of other historians who have delved into the archival materials and read through mountains of reports: (1) the science of risk assessment was politicized; (2) scientific groups at the national and international level limited the discussion of biotechnology to laboratory risks, excluding the social and ethical implications; (3) policy options among scientists were narrowed by self-interest.

The strongest features of this book are its discussions of international scientific networks and the role they played in the early debates over rDNA. However, it is primarily a discussion of events (not the people who shaped them) and formal relations among transnational institutions. Russell describes the patterns of interaction among scientists as "multilevel overlapping systems". His use of network analysis and organizational theory, unfortunately, does not deepen our understanding of the historical events. There is little substantive analysis of the effect that second-order (international) organizational structures had on the way that first-order (national) structures handled risks.

We learn from this study that the systems designed to promote scientific exchange and cooperation were highly successful in structuring the international debate over biotechnology. Moreover, the same scientific groups that were so effective in damping the regulatory oscillations for rDNA research have been noticeably silent on associated ethical dilemmas. We have heard little from them on such issues as American scientists using Third World nations as testing grounds for new vaccines, growing secrecy within the biological research community and the rise in world-wide military interest in biological weapons. □

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The good and the great

Stuart Sutherland

A Passion for Science. By Lewis Wolpert and Alison Richards. *Oxford University Press: 1988. Pp.206. £15. To be published in the United States in August, price \$21.95.*

SCIENTISTS lead an enviable life. They richly deserve to do so, for they are an exceedingly pleasant lot — or so it would appear from the remarks of the 13 interviewed by Lewis Wolpert for the BBC.

Their work is fun, a word that appears again and again. Indeed it is so much fun that some of them feel a little guilty about their self-indulgence. Few of them mention the weary weeks or months spent unsuccessfully trying to solve a problem or to make an experiment work, let alone the misery of discovering that someone else has got there first. Oddly, the issue of priority is hardly raised, though depending on which way it goes it probably causes more joy and heartbreak than anything else in science.

With the exception of Stephen Gould, they lack any trace of conceit: one cannot help wondering how such bashful virgins came to achieve such eminence. When awarded Nobel prizes, their immediate reaction is to think the Nobel Committee has written to the wrong chap. They modestly point out that chance played an important role in their most important discoveries. John Maynard Smith was prompted to invent the concept of an evolutionarily stable strategy and to carry out the mathematical analysis that underpinned it by reading an unpublishable paper that he happened to referee. And according to his own account, Francis Crick entered biology because he was told to do so by a physicist he knew during the war, and he met Jim Watson by accident.

The moral virtues displayed by Wolpert's scientists are remarkable. They do not steal ideas from their colleagues, though they may steal their wives from time to time. They never ever cheat in science, though we are not told whether they defraud the customs or the Inland Revenue. Nor do they pinch ideas without acknowledgement, even if they are sometimes tempted to pinch their *au pairs'* bottoms. And if they have ever had cantankerous arguments with fellow scientists, they do not mention them. It is all very bland. True, Sydney Brenner raises a smile when he recounts how he advised a student to stop Xeroxing articles. When he told him to "neurox" them instead, he had to explain what he meant. Neuroxing is "a very easy and cheap process. You hold the page in front of your eyes and you

let it go through there into the brain. It's better than Xeroxing".

Although it may be hard to believe the scientists' self-portraits, their accounts of their work are always clear and there is the occasional nugget of information. For example, Michael Berry explains that the inevitable uncertainty about the position of an electron at the known limit of the Universe will cause an error of up to 90° in the predicted direction of a gas molecule on Earth after it has collided only 50 times with other molecules, a process that takes a fraction of a microsecond.

One longs for more of this kind of thing rather than being repeatedly told that science is fun. Indeed the book's worst fault is that it shies away from proper explanations. There is a clear account by Anthony Epstein of how the Epstein-Barr



Sydney Brenner — in praise of the neurox.

virus was discovered: the reader is repeatedly told how important the discovery was, but he never learns why. It is true that the BBC usually aims to present material that will not stretch the intellect of a five-year-old, but the lack of more detailed expositions of what was found out and why it was important is frustrating.

It may be that anything more difficult would have driven listeners away. Wolpert himself argues in his introduction that for two reasons science is little respected in Britain and hence is understood by few. First, the romantic movement, at least in the shape of Coleridge followed by D.H. Lawrence, took an anti-reductionist stance. But engineering was widely admired in Victorian times, even by some poets like Tennyson and subsequently Kipling. Moreover, in the 1930s and 1940s names like Jeans and Eddington were household words, but if in a pub quiz today there was a question asking the name of any living astrophysicist, the quiz devotees would probably arise as one and drench the quiz master with Guinness for setting an unfair question. Wolpert's second argument also seems weak. He writes "the scientific mode of thought is neither natural nor comfortable". But it is

not so long ago that almost all children at school over (and often under) the age of 12 had to learn Latin and Greek. Surely classical languages are even less natural and comfortable than science — for the British that is. There is no reason to suppose that they presented much difficulty to the ancient Greeks or Romans.

Fashion is always hard to understand: you may just as well ask why over the past two years women have abandoned pale stockings for black ones as seek reasons for the abysmal ignorance of science in Britain. In deploring this state of affairs,

Wolpert is strongly supported by Walter Bodmer who points to the lack of scientists in government or administration: after all, they have “an approach to thinking about problems in a rational way”. Unfortunately neither of them proposes a solution, but if this book raises the image of the scientist in the eyes of the British public — as it certainly will if they believe what they read — it will have served a useful purpose. □

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Configuring it out

Rodney J. Bartlett

Methods in Computational Chemistry. Vol. 1, Electron Correlation in Atoms and Molecules. Edited by Stephen Wilson. Plenum: 1987. Pp.363. \$78, £43.35.

ANY COLLECTION of review papers on an active research area depends on two factors to distinguish it from an excess of similar volumes: the novelty of the topics addressed, and the presentation, scholarship and depth of the contributions. This book, the first in a new series, deserves high marks on both counts.

A defining characteristic of the articles is the emphasis on approaches to electron correlation which are usually called ‘many-body’ (that is, many-electron) methods. These methods include many-body perturbation theory (MBPT), coupled-cluster (CC) theory and propagator approaches, all of which are addressed at length in various chapters. Although no single review deals primarily with the oldest class of correlated methods, configuration interaction (CI) and its very modern unitary group implementations,

many of the contributions, and particularly that of Jankowski *et al.*, discuss the considerable contribution of modern CI.

Electron correlation in atoms, as opposed to molecules, frequently receives too little attention today from the quantum chemistry community. Jankowski restricts himself to atomic theory as studied by methods which (except for symmetry considerations) are also applicable to molecules. This review largely deals with contemporary thinking on the subject, but it also reminds the reader of the origins of a body of essential developments, now often forgotten.

Today, few research topics in electron correlation are expanding as rapidly as the coupled-cluster and their finite-order MBPT approximations, reviewed by Urban *et al.* New developments occur almost daily in this field, and this thorough review highlighting recent events and key references, and pointing the way towards future advances, is unusually timely. Particularly useful is the authors’ discussion of properties other than the energy in CC theory, including analytical derivatives. The thoroughness of the treatment, which includes many numerical results, strongly recommends this contribution.

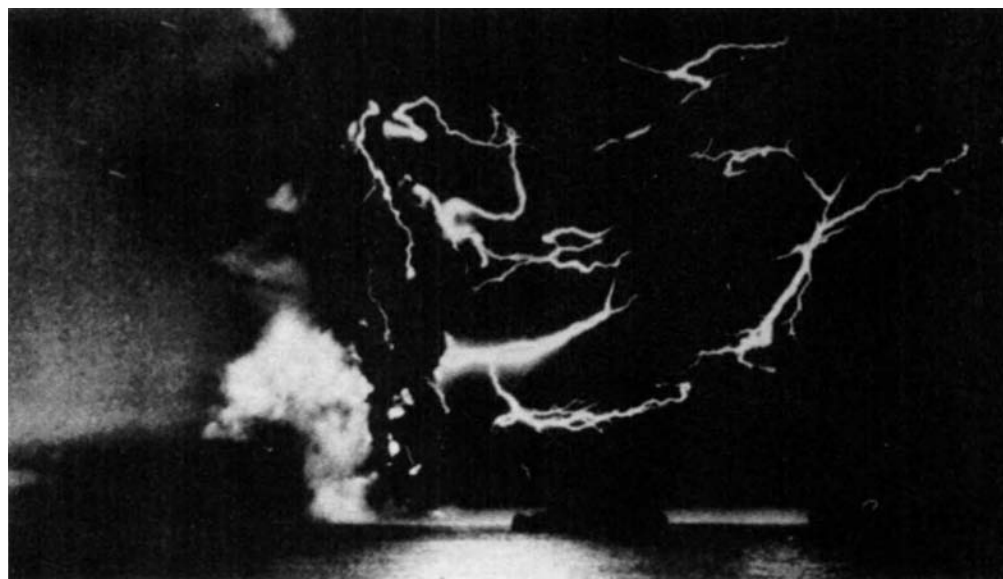
At the core of any correlated method

lies an integral transformation. The methods for doing such transformations have been known for some time, yet few authors have critically assessed the different approaches. One particularly important question addressed in the book is whether an integral transformation is the best strategy for many problems. Ways to avoid most of a transformation, such as the so-called coefficient matrix approaches, would be recommended for large-molecule calculations, for example.

Different approximations for electron correlation have their strengths and weaknesses, yet underlying any such method is another approximation, of perhaps greater importance, which might be termed the (primitive) basis set problem — or the limitation in describing the wavefunction for a molecule in terms of a limited number of atomic orbitals for each atom. Green’s function Monte Carlo (GFMC) methods potentially offer a way to avoid such basis set problems. For electrons, however, other kinds of approximation pertaining to the location of wavefunction nodes and the existence of vastly different ‘time’ scales for inner and valence electrons introduce problems that have proved difficult to resolve. Also, the extreme computational demands of GFMC are a limiting factor in applications to molecules. The article by Wells takes the reader through the developing GFMC area, delineating the ‘fixed-node’ and ‘short-time’ approximations, and how they may be removed.

Each of the reviews in this collection is well written and up to date. The book as a whole is well worth the attention of quantum chemists, and the high quality of the contents augurs well for future volumes in the series. □

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Flashes of brilliance — lightning in the volcano cloud over Surtsey, near Iceland, in December 1963. The November eruption resulted in the formation of an island nearly 1 kilometre long and 100 metres above sea level. The picture is taken from *The Lightning Discharge* by Martin A. Uman, just published by Academic Press as part of their *International Geophysics Series*. Price is \$49, £31.50.

Pseudoscience and antiscience

Lewis Wolpert

The New Age: Notes of a Fringe Watcher. By Martin Gardner. *Prometheus*: 1988. Pp.273. \$19.95, £14.95.

It is, as we all know too well, very difficult to get a paper published in *Nature*. A lack of interest, or hint of doubt by a referee can sway the decision towards rejection. Yet in October 1974 this journal published a report that Uri Geller was able to divine the face of a dice a million times better than would be expected by chance. Reviewing the evidence, Martin Gardner argues that the original experiments were poorly controlled and, like so many other cases, were unreliably anecdotal. *Nature's* willingness to publish such a paper may be an extreme example of the desire among many people to believe in the paranormal and allow themselves to be tricked. "As all magicians know, physicists are the easiest in the world to be fooled by magic tricks."

In this series of essays, about half of which were published in the *Skeptical Inquirer*, Gardner, who is best known for his writing on mathematical recreations, devotes himself to debunking pseudoscience. Debunking is a pejorative term used by the defenders of the paranormal, but Gardner, following Stephen Gould, sees it as essential to the health of science. Many of the essays are devoted to exposing the fraud in claims, for example, for N-rays, spiritualism and psychic surgery. The essay on the magic number 666 is great fun. With $A = 100$, $B = 101$, Hitler adds up to 666.

A distinction should be made between pseudoscience and unconventional science. Gardner uses as an example the work of the astrophysicist Thomas Gold, whose career, he suggests, is a thousand times more interesting and significant than that of a crank like Velikovsky. Gold was brilliantly correct about pulsars but his views on the surface of the Moon, and more recently his non-organic theory of the origin of oil and natural gas, are totally unacceptable to most geologists. There is a natural and justifiable hesitation by scientists to accept unconventional ideas. But this should not be confused with the rejection of pseudoscience where there are neither data nor connections with the rest of science.

Examples of pseudoscience are Sheldrake's invocation of a quite new set of mystical forces, 'morphic resonance', to explain embryonic development, and claims for levitation or mind-reading. It is not just that the evidence is not available but there is only the flimsiest connection with current science. Perhaps a further

distinction should be made within pseudoscience itself between those who are honourable and absurd, like Sheldrake, and those who are just fraudulent like Geller.

A threat to science comes less from the absurdities of the paranormal than from the sociologists of science. It is easy to dismiss a channeller who first made contact with Ramtha, who was born 35,000 years ago in the slums of Atlantis, when she put a model of the Great Pyramid on her head. Less easy to deal with, and more insidious, is the anti-science relativism of Feyerabend and Collins and Pinch. The latter, in their discussion of paranormal metal-bending have adopted a relativist position. Gardner, rightly, is shocked by their total indifference to whether the phenomenon exists or not. For them there is no rational means of evaluating conflicting claims. Just because there is no clear

way of demarcating between science and non-science should not prevent us from recognizing the absurd when we see it.

There are essays on perpetual motion, creationism and fundamentalist preachers, but as a whole, the book does not work. Though well written and witty, some of the pieces are too slight and the topics too parochial. Many of the targets are too easy. The book does not come to grips with the important issues as to why pseudoscience flourishes, whether it matters, and what we can do about it. In the end, stories about metal bending and Shirley MacLaine's channelling boy-friends become boring. □

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Cramp continues

Jeremy J.D. Greenwood

Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic. Vol. V, Tyrant Flycatchers to Thrushes. Editor-in-chief Stanley Cramp. *Oxford University Press*: 1988. Pp.1,075. £75, \$175.

STANLEY Cramp, who died last year, was a leading figure in British ornithology — chairman of numerous committees, campaigner for conservation, driving force in establishing the Seabird Group, senior editor of *British Birds*. Amid all these activities, it is surely his editorship of 'BWP' that will ensure his place in history. There can be few serious ornithologists or respectable libraries in Europe that do not purchase each volume as it becomes available.

What can one say of the latest addition to such a well-known work, except that it is another element in a landmark in ornithology? The format of Vol. V is similar to that of earlier volumes, although, in keeping with the expansion of ornithological knowledge, there is 47 per cent more text per species than in Vol. I. The behaviour sections have continued to be more functional and less merely descriptive. The introductory material from Vol. I is presented again, with some revisions, especially concerning behaviour and habitat. There is still too little, however, on the methods used to obtain and present the information — for example, the difficulties of making repeatable measurements of birds and of estimating survival rates are not mentioned — and, overall, few of the criticisms made by reviewers of previous volumes have been met. Of course, those criticisms are minor in

relation to the monumental significance of the work, and one can see that the failure to respond might be justified by the need to keep the presentation uniform throughout the volumes. But one hopes that they will be heeded when the second edition is produced.

My main personal disappointment is the unsatisfactory treatment of eggs. No standard deviations of measurements are given; nor are egg volumes. The expression 'calculated weight' is not explained and rates of loss of weight during incubation are not provided. Shape is described in terms that are said to be "self-explanatory", a phrase which nearly always means that the writer is unwilling to be tied down to precise definitions. In a work such as this, terms should be defined clearly; even better would be to give, as well as length and breadth of eggs, the distance of the broadest point from the ends. The illustrations, too, are poor. Because of the wrong choice of background colour, some white eggs are almost invisible. And most of the photographs are badly illuminated: even the Dunnock's egg, in reality a heart-stoppingly bright blue, appears dull. Although a range of variants is shown for the eggs of most species, the relative frequencies of the variants are not.

At the present rate of progress, Vol. VII of BWP will appear 17 years after Vol. I, which will thus be substantially out of date. Could thought now be given to the idea of a continuing cycle of revision, one volume every three years say, so that new editions of each volume appear every 21 years? Such regular revision would be a great service to ornithology — and a continuing memorial to Stanley Cramp. □

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Causes of rapid motions of the Earth's pole

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Analysis of new highly accurate geodetic data reveals rapid motions of the Earth's pole, with peak-to-peak variations of ~0.002 to 0.020 seconds of arc, fluctuating on timescales between two weeks and several months. Comparison with meteorological excitation estimates shows that these motions are at least partially driven by surface air pressure changes as modified by the response of sea level to atmospheric loading. Such geodetic measurements thus potentially provide a novel means of observing the dynamics of the atmosphere and oceans at very low spatial wavenumbers.

THE Earth does not rotate perfectly uniformly, but undergoes relatively small variations in rotation rate and in the direction of its rotation axis; such changes are monitored through observations of extraterrestrial objects from the surface of the Earth. Motions of the rotation axis are caused by exchanges of equatorial angular momentum between the 'solid' Earth (the crust and mantle) and its environs and by deformations of the solid Earth, and geodetic observations of such motions can potentially provide information about a wide variety of geophysical phenomena^{1,2}. Changes in the direction of the pole are divided into precession, nutation and polar motion, with the first two terms describing motions of the rotation pole relative to an inertial frame, and the third motions of the rotation pole relative to the crust. This report concerns polar motions that are fast compared with those previously observed, but still slow compared with the diurnal cycle.

The relation between the observed polar motion and its excitation can be concisely expressed in complex notation using an Earth-fixed coordinate system^{1,2}. It is assumed that the crust and the mantle rotate together after accounting for tectonic motions. Let the coordinates of the rotation pole along axes pointing towards the Greenwich meridian and 90° E longitude be denoted by m_1 and m_2 , respectively. The excitation, denoted by χ_1 and χ_2 , is proportional to the components of terrestrial angular momentum about those same axes. The response of the Earth to such excitation is, to a good approximation, given by

$$\frac{dm}{dt} = i\sigma(m - \chi) \quad (1)$$

where m is ($m_1 + im_2$), σ is $(1 + i/2Q) \times 2\pi/T_c$, and χ is ($\chi_1 + i\chi_2$). The complex resonance frequency is represented by σ with T_c being the Chandler wobble period (~433 days) and Q the dimensionless quality factor (generally thought to be >100, and thus large enough for dissipation to be ignored for our purposes).

The resonant oscillator described in equation (1) strongly amplifies the effects of eastward propagating (or prograde) excitations with rotational periods near T_c . The possibility of observable polar motions resulting from this wobble resonance was first noted by Euler in 1765, and the forced prograde annual wobble, greatly amplified by the resonance, as well as the resonant motion itself (the Chandler wobble), have been under continuous observation and study since the work of Chandler in the 1890s^{1,2}. Excitations at higher frequencies, further from the wobble resonance, are strongly attenuated, and the existence and nature of faster polar motions have long been in doubt. Two new techniques, very long baseline interferometry (VLBI) and satellite laser ranging (SLR), now monitor the polar motion on a regular basis with a demonstrated accuracy of ~2 milliarcseconds (or mas) in both components of polar motion^{3,4}, a

factor of 5–10 times more accurate than earlier techniques⁴. The past decade has also seen the development of global numerical weather analysis and forecasting, enabling the production of accurate atmospheric angular momentum estimates on a regular and frequent basis⁵. These new data types are sufficiently accurate to permit the study of more rapid polar motions.

The existence of small rapid changes in the path of the pole has recently been shown using VLBI and SLR data (refs 3, 4 and 6). Rapid changes in equatorial atmospheric angular momentum estimates are also observed^{5,7,8} and have previously been related to polar motion changes⁵, although not in depth. We use a correlational analysis of geodetic data and components of meteorological excitation estimates to show that the observed rapid polar motions are related to atmospheric pressure changes, and that the oceanic response to atmospheric pressure loading is an important component of the excitation budget.

Geodetic excitation estimates

The accuracy and precision of global geodesy have greatly increased recently as older techniques, based on mechanical measurements of angles, have been supplanted by methods based on electronic measurements of time delays and frequency shifts. A recent international programme to monitor Earth rotation and intercompare techniques greatly stimulated monitoring of the polar motion, especially during and after the main measurement campaign (September 1983 to November 1984)⁹.

Geodetic excitation estimates used here were derived from a combination of one SLR solution and three separate VLBI efforts (manuscript in preparation). Polar motion estimates from laser ranging to the LAGEOS satellite were part of the University of Texas Center for Space Research solution LLA 8511, which provides five-day averages of the polar motion starting in May 1976, and three-day averages starting in July 1985¹⁰. The International Radio Interferometric Surveying (IRIS) project began a regular programme of multibaseline Europe–America VLBI experiments at the beginning of 1984, typically with a 24-h duration session scheduled every five days; single and multibaseline results are also available for 1983. We used polar motion estimates from a reduction of IRIS data by the US National Geodetic Survey (solution 3/15/87)³. The US Deep Space Network (DSN) conducts single baseline VLBI observations over Spain–California and Australia–California baselines, typically on a weekly basis; most of these observing sessions have durations of three hours although there were also some observing sessions of longer duration (18 to 24 h) during 1984 (ref. 4). Further occasional single and multibaseline VLBI results were provided from observations conducted by the NASA Crustal Dynamics Project; these data were reduced by the Goddard Space Flight Center¹¹.

In equation (1), the polar motion arises from the linear convolution of a complex (two-dimensional) excitation vector with the impulse response of the Chandler wobble resonance¹². Com-

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parison of the geodetic and meteorological data thus requires either the convolution of the meteorological excitation data or the deconvolution of the geodetic polar motion data⁸. Because the wobble has a very sharp resonance with a damping time of decades, deconvolution of the polar motion data is preferred as a means of prewhitening¹². Direct numerical deconvolution greatly amplifies any measurement noise at high frequencies, and thus is typically accompanied by some sort of smoothing, usually in the frequency domain^{13,14}. The geodetic data, however, are not uniformly accurate or available at regular intervals, a severe impediment to frequency domain techniques, and we adopted a time-domain Kalman filter for deconvolution.

The JPL Kalman filter was developed to smooth and predict earth orientation changes in support of spacecraft navigation by the DSN, and yields deconvolved excitation estimates as a by-product¹⁵. The results from two filters, one moving forward and the other backwards in time, are combined to provide smoothed excitation estimates. The filter uses stochastic models to account for excitation changes between measurements, and thus varies the amount of smoothing according to the quality and density of the geodetic data. Spectral analysis of Kalman-smoothed excitation estimates shows that the Kalman deconvolution is equivalent to a direct deconvolution followed by a low pass filter. The low pass filter gain (for the modern data) is 0.5 at a period of ~ 17 days in both χ components and falls smoothly to zero at shorter periods¹⁵. Any induced shifts in phase are negligible, and the Kalman deconvolution is empirically found not to introduce spurious fluctuations into the smoothed data. We do not claim here that our deconvolution method is necessarily optimal, but it is suitable for our purposes.

Meteorological excitation estimates

It has long been clear that redistributions of atmospheric mass play a role in both the annual and Chandler wobbles¹, but the examination of more rapid meteorological effects has only recently been made possible by modern weather analysis and forecasting techniques⁵. Such techniques rely on assimilating large quantities of meteorological data into numerical atmospheric models. These efforts provide estimates of the current atmospheric state at a dense set of grid points throughout the atmosphere¹⁶; calculation of the atmospheric excitation by the weather centres uses these analysis fields, typically available daily or twice daily.

The total atmospheric angular momentum vector (or χ vector) can be divided into two equatorial components (χ_1 and χ_2) associated with the excitation of the polar motion, and an axial component (χ_3) involved with changes in the length of day⁵. Each component can, to a good approximation, be further separated into so-called pressure and wind terms, which we denote by χ^p and χ^w respectively. Barnes *et al.*⁵ provide a complete derivation of both the pressure and wind terms of all three χ vector components; their expressions for χ_1^p and χ_2^p are (in mas)

$$\begin{aligned} \begin{bmatrix} \chi_1^p \\ \chi_2^p \end{bmatrix} &= -2.063 \times 10^8 \times \frac{1.00 R^4}{(C-A)g} \int_0^{2\pi} \int_{-\pi/2}^{\pi/2} p \sin(\phi) \\ &\quad \times \cos^2(\phi) \begin{bmatrix} \cos(\theta) \\ \sin(\theta) \end{bmatrix} d\phi d\theta \end{aligned} \quad (2)$$

where the integral is over the entire surface of the Earth, R is the mean radius of the Earth ($\sim 6.37 \times 10^6$ m), $(C-A)$ is the difference of the polar and equatorial moments of inertia of the crust plus the mantle ($\sim 2.34 \times 10^{35}$ kg m²), p is the local surface pressure, g is the acceleration due to gravity (9.81 m s⁻²), and ϕ and θ are the latitude and East longitude, respectively. The numerical factor of 1.00 in equation (2) represents the net effect of solid Earth deformations induced by atmospheric pressure loads and by rotational deformations⁵. Equation (2) agrees numerically with the analogous equation of Wahr¹⁷ to better than 1%, although Wahr considers directly the effects of the

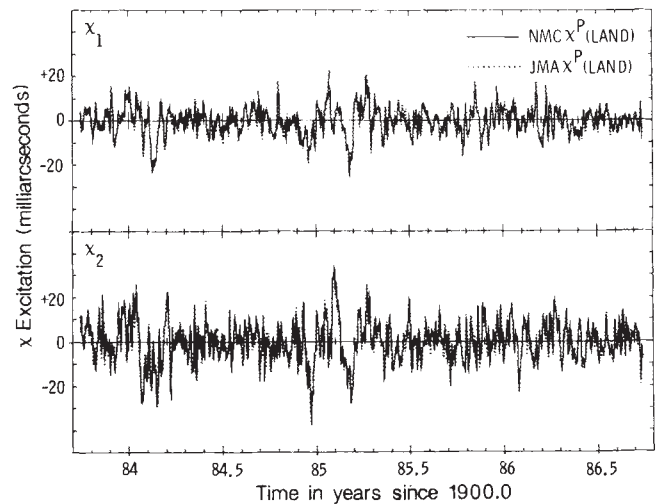


Fig. 1 Determinations of χ^p (land) from the NMC (solid lines) and the JMA (dotted lines), after seasonal adjustment.

static pole tide in the ocean and treats the response of the liquid core differently. Equation (2) can be used to show that χ_1^p and χ_2^p are proportional to cosine and sine components, respectively, of the P_2^1 spherical harmonic of surface air pressure¹. Changes in χ^p thus represent zonal wavenumber one surface pressure changes that are antisymmetric about the equator, with an increase in χ of one mas being equivalent to a P_2^1 surface pressure change with a maximum of ~ 4 kg m⁻¹ s⁻² (or 0.04 millibars) at latitudes of 45° North and South, and to an atmospheric angular momentum change of $\sim 8.3 \times 10^{22}$ kg m² s⁻¹. The wind term, χ^w , (described in ref. 5) corresponds to rigid body rotation of the atmosphere about an axis in the equatorial plane, with a χ^w variation of 1 mas corresponding to a maximum velocity change of 0.0026 m s⁻¹.

Changes in the surface air pressure over the oceans cause load-induced displacements of water that modify the excitation caused by surface pressure variations. This effect is generally modelled by assuming that the oceans are able to reach equilibrium with the surface loading and thus behave as a perfect inverted barometer, with any changes in the local air pressure being almost exactly compensated by opposing changes in the local sea level^{2,17}. In this model the oceans will exchange angular momentum with the atmosphere, reducing the exchange between the atmosphere and the solid Earth. To investigate possible failures of the inverted barometer model at high frequencies, we used the available χ^p data, with and without this correction, to estimate the contribution of surface pressure changes to χ^p separately over land and over the oceans; we denote these data by χ^p (land) and χ^p (ocean), respectively. χ^p (land) is thus the effect of pressure changes after application of an inverted barometer model, whereas χ^p (the sum of χ^p (land) and χ^p (ocean)) ignores the inverted barometer response, effectively assuming a rigid ocean. (The calculated χ^p (ocean) does not include changes in the average surface pressure over oceanic grid points, but this error is only a few per cent of the total effect.)

Our study was conducted with excitation estimates from the global analysis fields of the US National Meteorological Center (NMC) and the Japanese Meteorological Agency (JMA)¹¹. These centres calculate both wind velocity and surface pressure fields, and so both data sets include the pressure and wind terms for each component; the inverted barometer corrections are also available from both centres. All the meteorological data used were sampled twice daily, at noon and midnight UTC, although much of the JMA data were available only in the form of daily averages. The wind data contain a strong diurnal variation, and, to avoid aliasing problems, all the twice-daily meteorological estimates were similarly converted into daily averages. As the

seasonal cycle was not the subject of our analysis and could corrupt our statistics, we seasonally adjusted all the data used in this study by removing a best-fit bias and annual and semi-annual sinusoids from each data set. We limit our analysis to exactly three calendar years (1,096 days) of data from September 1983 to September 1986, to take advantage of the most accurate geodetic and meteorological data and to simplify the seasonal adjustment. There were 131 missing excitation vectors in the twice-daily NMC data set for the analysis period; these gaps were filled by linear interpolation. A discontinuity in the NMC pressure data after 28 May 1986, due to changes in the NMC orography field, was removed by estimating a separate bias for the NMC data after that date.

Analysis of meteorological estimates

Comparison of excitation estimates from the two weather centres was used to assess the reliability of the atmospheric data. Figure 1 presents the daily χ^p (land) estimates from the NMC and the JMA after seasonal adjustment. Rapid oscillations are clearly evident in Fig. 1, as is the close agreement of the pressure terms from the two weather centres. Table 1 presents (at the top) the standard deviations of the NMC data after seasonal adjustment (similar results are obtained from the JMA data). The rapid variations in the total pressure and wind terms are roughly isotropic, with the standard deviations of the total χ^p being larger than for χ^w , and with most of the χ^p power being in χ^p (ocean). The observed variations in χ^p are equivalent to root mean square (r.m.s.) surface pressure changes of ~ 1.4 millibars at the latitudes of maximum effect, and the χ^w changes correspond to maximum r.m.s. wind velocity changes of $< 5 \text{ cm s}^{-1}$.

The linear cross-correlation between series was used to assess the statistical significance of relationships in this analysis. The autocorrelation functions of the seasonally adjusted meteorological data sets drop rapidly with increasing lag, falling below 0.5 after lags of 3 days or less. We estimate that the 99.9% confidence threshold for a test of independence using three years of these data is ~ 0.2 , based on a null hypothesis of no correlation, the observed autocorrelations, and the correlation variance estimates of Bartlett¹⁸. Table 2 shows that every correlation between the seasonally adjusted NMC and JMA χ^p (land) and χ^p (ocean) is greater than 0.9 at zero lag, which is statistically strong evidence of dependence, and there is a weaker but still significant correlation of ~ 0.4 between the seasonally adjusted χ^w . (No significant peaks were observed away from zero lag in any of the cross- or autocorrelation functions examined in this study.) Another measure of the agreement of two time series is the r.m.s. scatter of their difference, also shown in Table 2. A comparison of Tables 1 and 2 shows that, although the r.m.s. differences of the JMA and NMC pressure terms are small compared with the r.m.s. of the pressure terms themselves, this is not true for the χ^w , which is further evidence of the relatively large errors in those data^{7,8}.

Table 1 Standard deviations of the seasonally adjusted excitation estimates

Data set	Standard deviation (mas)	
	χ_1	χ_2
Meteorological data before smoothing		
NMC χ^p (land)	12.9	18.9
NMC χ^p (ocean)	30.9	28.0
NMC χ^p	35.1	35.4
NMC χ^w	17.5	17.8
Kalman smoothed data		
Combined χ^G	16.8	26.1
NMC χ^p (land)	9.0	12.9
NMC χ^p (ocean)	19.2	19.0
NMC χ^p	21.4	22.5
NMC χ^w	8.6	8.3

Table 2 Agreement of seasonally adjusted series

Data set	r.m.s. difference (mas)		Cross- correlation coefficient*	
	χ_1	χ_2	χ_1	χ_2
Meteorological data before smoothing				
NMC versus JMA χ^p (land)	2.7	3.9	0.98	0.98
NMC versus JMA χ^p (ocean)	12.1	13.1	0.92	0.90
NMC versus JMA χ^w	18.4	19.7	0.45	0.42
Kalman smoothed data				
VLBI versus SLR χ^G	9.8	10.4	0.81	0.91
NMC versus JMA χ^p (land)	1.5	2.2	0.99	0.99
NMC versus JMA χ^p (ocean)	6.8	8.0	0.93	0.91
NMC versus JMA χ^w	7.7	9.2	0.60	0.52
χ^G versus NMC χ^p (land)	15.1	20.7	0.45	0.62
χ^G versus NMC χ^p	22.2	23.7	0.34	0.53
(χ^G - NMC χ^p (land)) versus NMC χ^w	15.6	21.4	0.22	0.12
versus NMC χ^p (ocean)	22.2	23.7	0.18	0.29

* Values > 0.2 (before smoothing) and 0.3 (for smoothed data) are significant at the 99.9% level (see text).

The rapid χ^p (land) changes visible in Fig. 1 are not strictly periodic and cannot be explained by astronomical forcing. Similar global variations in surface air pressure have been observed previously^{19,20}; meteorological studies of these planetary pressure waves frequently assume that such global surface pressure changes result from excitation of atmospheric normal modes²¹⁻²³. The longest wavelength barotropic normal modes are, at least in theory, each composed predominantly of only a few low-order surface spherical harmonics^{20,24}, and the χ^p variations are closely related to one observed normal mode²⁵, called H_1^1 (ref. 19), R_1^1 (ref. 21) or simply (1, 2) (refs 22 and 23). Using the NMC analysis fields for July 1976 to mid-October 1979, Ahlquist claimed to detect this mode with an estimated period of 8 to 20 days, an estimated average amplitude of 0.6 millibars at $\pm 45^\circ$ latitude²², and a considerable amplitude variation with time²³ (see also refs 20, 21).

Comparison of excitation estimates

Separate Kalman deconvolutions of the SLR and VLBI polar motion data show common rapid fluctuations, which, when converted back into polar motions, have standard deviations during the analysis period of 11 mas (for m_1) to 6 mas (for m_2). These variations, although small, are larger than the proven 2 mas accuracy of the modern polar motion data^{3,4}, and their reality is supported by further analysis²⁵. The autocorrelation functions of the Kalman smoothed data do not fall below 0.5 until lags of ~ 7 days, and the 99.9% confidence threshold for a test of independence using three years of these data is therefore ~ 0.3 , using Bartlett's formula as before¹⁸. The observed correlation of > 0.8 between the seasonally adjusted SLR and VLBI excitation estimates during the analysis period (see Table 2) is thus strong statistical evidence for the existence of similar rapid polar motions in both geodetic data sets. The χ excitation functions were then estimated for each day in the analysis period from a Kalman deconvolution of the combined geodetic data set to provide the best possible geodetic excitation estimates; these are denoted by χ^G .

Direct comparison of raw and smoothed data can be misleading^{8,12}, and so the meteorological data were smoothed by convolution with equation (1) and deconvolution with the Kalman filter; care was taken to apply exactly the same smoothing to the geodetic and meteorological data. After seasonal adjustment, the deconvolved data contain significant power at periods between ~ 14 and 45 days, and therefore our further analysis is sensitive to oscillations in this period range. Table 1

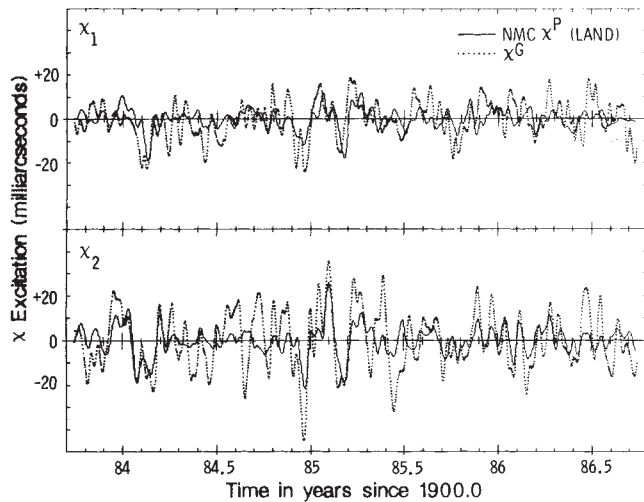


Fig. 2 Kalman smoothed estimates of the NMC χ^p (land) data shown in Fig. 1 (solid lines) together with the Kalman deconvolved χ^G (dotted lines) after seasonal adjustment.

shows that the standard deviations of the smoothed χ^p (land) and χ^w data are smaller than those of the smoothed χ^p (ocean) data. Results of comparisons of smoothed data are contained in Table 2, which shows that smoothing raises the correlation between the NMC and JMA data and considerably reduces the scatter between these series. Note that the agreement between the smoothed NMC and JMA χ^p (land) series is considerably better than the agreement between the two geodetic techniques, whereas the agreement between the other meteorological series is roughly comparable to the agreement between the geodetic series. In the rest of the paper we will concentrate on the NMC data as very similar results were found using the JMA data.

Figure 2 shows the Kalman deconvolved geodetic data, together with smoothed NMC χ^p (land), after seasonal adjustment. It seems clear from this figure that there is some relationship between the deconvolved geodetic and meteorological data; this conclusion is supported by the results presented in Table 2. The 99.9% confidence limit for a test of significance is again ~ 0.3 , and the correlation observed between χ^G and χ^p (land) is thus significant for both χ components (see Table 2). Addition of the atmospheric wind data to the χ^p (land) does not significantly improve the agreement with the geodetic data, and there is no significant correlation between χ^G and the smoothed χ^w , even after subtraction of the χ^p (land) changes (Table 2).

There is evidence of significant additional excitation besides the measured χ^p (land) and χ^w , especially in χ_2 . Table 2 contains the r.m.s. scatter between the SLR and VLBI χ estimates and also between the NMC and JMA atmospheric χ estimates, which provide some indication of the level of error in each data set. If it is assumed that the errors in each data set are uncorrelated, that independent measurements of the same series have roughly equal errors, and that there is no other source of excitation, then these r.m.s. scatters imply that the geodetic and atmospheric χ data should agree to within about 9 to 10 mas r.m.s., significantly less than the observed 16 to 21 mas r.m.s. scatter. There is no evidence that one meteorological or geodetic series is significantly better than the other, and so these results imply either a large common error in one or more of these series, or another source of excitation. The 'missing' excitation, of whatever source, has an approximate r.m.s. of 13 mas (for χ_1) and 19 mas (for χ_2), and is thus larger than the standard deviation of either the smoothed χ^p (land) or the smoothed χ^w . (The correlations between the various difference series are small enough not to significantly affect any of these error budget calculations.)

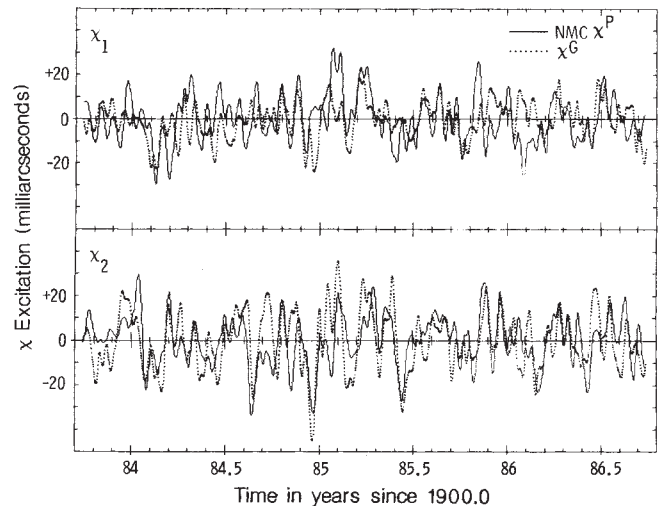


Fig. 3 Kalman smoothed estimates of the NMC total χ^p (solid lines) with the geodetic data from Fig. 2 (dotted lines) after seasonal adjustment.

Sufficient power is observed in χ^p (ocean) (see Table 1) to make changes in ocean bottom pressure (the true pressure excitation from ocean regions) a possible source of the missing excitation. Pressure changes over the ocean cannot supply the missing excitation for an equilibrium ocean response because errors between our inverted barometer model and the self-gravitating, loading, static response are thought to be no more than 10%^{17,26}. The equilibrium model must fail, however, at sufficiently high frequencies as the diurnal ocean tide response is known to be far from equilibrium²⁷. The frequency of the transition to a nonequilibrium response is at present known only for a few localized areas²⁸, although numerical model results suggest that the global P_2^1 ocean response is within 10% of equilibrium at a period of 28 days²⁹. One possible nonequilibrium ocean response model, the rigid ocean model, can be evaluated simply by comparing the geodetic data with χ^p . Use of these data is found to slightly reduce the observed agreement (see Table 2), and the observed r.m.s. difference between χ^p and χ^G (~ 23 mas) is still considerably larger than the total expected error. If the inverted barometer model is correct, then there should be no correlation between χ^p (ocean) and the geodetic data; some correlation, at least in χ_2 , is observed after removal of χ^p (land) changes (Table 2), but it is only marginally significant at the 99.9% level. Although the rigid ocean model can apparently be ruled out, a dynamic ocean response could be quite complicated²⁷ and not simply correlated with χ^p (ocean), and thus cannot be ruled out. Figure 3 shows the Kalman deconvolved and seasonally adjusted NMC χ^p (namely, the total pressure term), together with the geodetic data from Fig. 2. Although inclusion of the χ^p (ocean) data appears to have generally worsened the agreement between the χ_1 components, the agreement in χ_2 is at times better with the χ^p (ocean) data than without, and again a dynamic ocean pressure load response cannot be ruled out.

Other potential sources of the missing excitation during the analysis period include systematic errors, mass redistributions in the hydrosphere, seismic excitations, and even fluid motions in the core; of these contenders, only seismic excitations can be firmly ruled out. Although the procedures and software used by the NMC and the JMA are largely independent (and similar results are obtained using data from the European Centre for Medium Range Weather Forecasts^{7,8}), these centres use essentially the same raw weather data, and it is not possible to exclude the existence of common errors, caused, for example, by lack of data from sparsely observed regions such as the South Pacific.

Aliasing of any nearly semidiurnal excitation variations will also introduce systematic errors, but this problem cannot be addressed given the available data. Redistributions of water on and over the continents are important in the excitation of the annual wobble³⁰, and more rapid redistributions could supply the missing excitation, as could fluid motions in the outer core, but little is known about these forcings at the periods of interest to this study^{31,32}. By contrast, moment of inertia changes associated with earthquakes do not appear to be important in the excitation of rapid polar motions, as the observed variations are larger than those expected from even the largest earthquakes³³, and a number of recent studies have found no noticeable relation between the recently observed polar motions and the smaller recent seismic events^{3,14,33}.

Conclusions

Rapid polar motions exist and are significantly correlated with one harmonic of global scale changes in the surface air pressure, but the contribution of atmospheric winds at high frequencies remains unverified. The correlation is greatest for pressure changes over land; the observed χ^p variations correspond to polar motions of roughly 6 mas (for m_1) and 3 mas (for m_2).

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A point mutation in the last intron responsible for increased expression and transforming activity of the c-Ha-ras oncogene

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The T24/EJ allele of the Ha-ras proto-oncogene owes its powerful oncogenic activity not merely to the well documented mutation that perturbs the structure of the encoded polypeptide, but in addition to a second single nucleotide alteration in an intron that causes a tenfold increase in expression. This effect on expression is maintained upon transfer of the surrounding DNA to a heterologous gene, and as such defines a novel regulatory element.

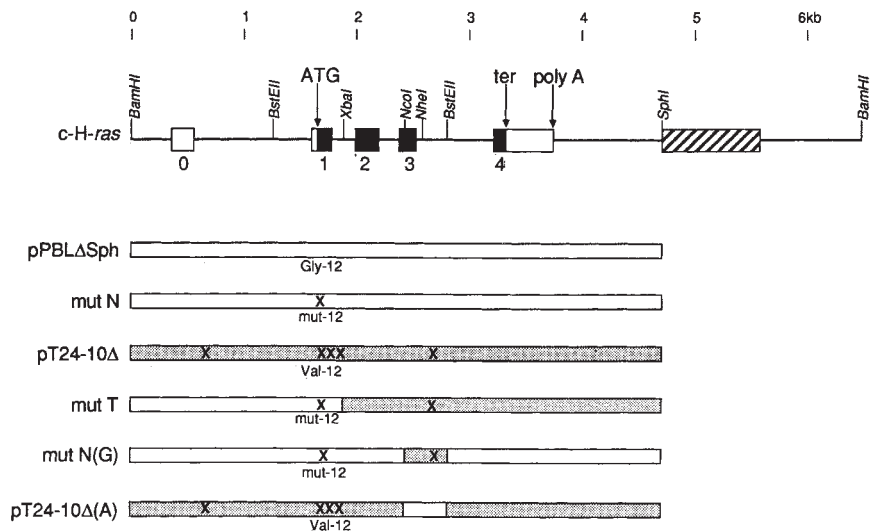
THE transforming human Ha-ras gene from the T24/EJ bladder carcinoma cell line has served as a prototype for that class of cellular genes (proto-oncogenes) whose mutationally altered product or aberrant expression can lead to the malignant transformation of non-tumorigenic cells^{1–3}. This was the first cell-derived oncogene whose mechanism of activation was elucidated at the molecular level. While nucleotide sequence comparisons identified a small number of differences between this

The geodetic effect of atmospheric pressure changes over the oceans appears to be strongly modified by the response of sea level to pressure loading, and the modern geodetic and meteorological data can thus be used to study the large scale pressure load response of the world ocean. Our results are consistent with an equilibrium inverted barometer ocean response model, but the atmospheric excitation data assuming that model do not fully account for the observed rapid polar motions, especially for the χ_2 component. Possible sources of the missing excitation are nonequilibrium ocean responses to atmospheric loading and hydrospheric mass redistributions. The observed rapid polar motions are large enough to severely complicate the detection of polar motion changes after large earthquakes.

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gene and its normal, non-transforming counterpart^{4,5}, a single such change, a point mutation that alters the amino acid encoded by codon-12, was shown to be responsible for the activation of its transforming potential^{5–8}. The T24/EJ gene has been extensively compared with one of several independently isolated normal cellular Ha-ras genes to study the effects of these two alleles on a broad array of biological and biochemical processes^{1–3,9}, including the oncogenic transformation of primary and

Fig. 1 Structure of the human c-Ha-ras gene and hybrids between normal and mutant forms. Upper, schematic representation of the gene⁵. Black boxes, translated sequences; open boxes, untranslated exon sequences. The hatched box flanking the *Sph*I site marks a polymorphic region of tandem repeats. Initiation (ATG) and termination (ter) codons and the polyA addition site are indicated. Only those restriction endonuclease sites relevant to the construction of hybrid genes are shown. Below, relationship between normal, T24/EJ and hybrid c-Ha-ras genes. Sequences derived from the normal gene present in plasmid pPBLΔSph (ref. 5) are indicated by open bars and those derived from the mutant gene cloned from the T24 bladder carcinoma cell line and present in plasmid pT24-10Δ (ref. 5) by shaded bars. Alterations relative to the normal allele are indicated by crosses⁵. As a class, genes containing a codon at position-12 other than for glycine or valine are termed 'mut'. The generation of *mut*-12 variants of plasmid pPBLΔSph by mutagenesis on a *Bst*EII-*Xba*I fragment has been described²⁰. Hybrid plasmids were constructed by conventional recombinant procedures⁴⁵.



established cells, cellular responses to a variety of growth factors^{10,11}, second messenger turnover (for example, cyclic AMP and phosphoinositides), gene expression¹², proliferation, differentiation and metastasis. Differential effects in all such cases have been interpreted to be a direct and unique consequence of the single amino acid difference at position 12 of the c-Ha-ras-encoded polypeptide, p21 (ref. 3); this is in contrast to another class of cellular genes (such as *myc* and *mos*) that manifest oncogenic potential when expressed at an inappropriate level^{1,2}.

Several lines of evidence indicate that the degree of cellular transformation by a structurally altered p21 is a function of the amount of this protein in the cell. For example, progressively higher rates of p21 synthesis controlled by an inducible promoter correlate well with an increasingly transformed phenotype of the cell¹³. Also, the highly efficient expression of retroviral oncogenes is thought to account for the potent oncogenic activity of acutely transforming retroviruses. In addition, overexpression of the normal cellular Ha-ras gene can result in transformation of NIH3T3 cells^{14,15} and, to a lesser extent, Rat-1 cells¹⁶. These observations suggest that *ras* gene expression must be tightly controlled, and that perturbations in this control could have significant consequences. We report here the first example of such a perturbation caused by a second mutation present in the T24 allele of the c-Ha-ras gene and demonstrate that this mutation acts to increase Ha-ras expression from a position not previously implicated in the control of this or other genes.

Intron mutation increases transformation

The human cellular Ha-ras gene consists of five exons located between *Bam*HI and *Sph*I restriction endonuclease recognition sites 4.7 kilobase pairs (kb) apart^{4,5} (Fig. 1). Exons 1 to 4 contain the coding information for the Ha-ras gene product, p21; the first exon (exon 0) is not translated^{5,17}. All genetic information required for expression of the gene, including its promoter and polyadenylation site, is present within the 4.7 kb of DNA (refs 5 and 7). An extensive array of tandem repeats specifying weak enhancer activity is located beyond this region 3' of the polyadenylation site^{18,19}.

We have been studying the biological properties of Ha-ras genes activated by a variety of *in vitro* generated mutations at codon-12 in exon 1 (ref. 20). The gene constructs used to this end differ from the normal cellular Ha-ras gene present in plasmid pPBLΔSph by just the codon-12 mutations and are identified here by the character N following the amino acid

encoded at position-12. In the course of this work we observed that replacement of the *Xba*I-*Sph*I fragment spanning exons 2 to 4 (Fig. 1) by the corresponding fragment present in our T24/EJ oncogene clone (plasmid pT24-10Δ) resulted in a marked (~tenfold) increase in the transforming activity of the codon-12 mutant genes as determined by their ability to induce foci on a monolayer of Rat-1 cells. Examples of these findings are presented in Table 1, where the chimaeric mutant genes include the character T in their designation. The results suggested that differences must exist between the normal gene and the T24/EJ oncogene in the region flanked by the *Xba*I and *Sph*I sites. Comparison of the complete published nucleotide sequence⁵ of both genes established that a single nucleotide difference distinguishes this region in one gene from that in the other. This difference—an A in the normal gene versus a G in the T24 gene—is located in the fourth intron (intron-D) at position 2,719 (numbered according to ref. 5), approximately 180 nucleotides 3' of the third coding exon and 520 nucleotides 5' of the fourth coding exon (Fig. 1). We determined the entire nucleotide sequence in plasmids trp12N and trp12T of the 430-base pair (bp) *Nco*I-*Bst*EII fragment that includes position

Table 1 Rat-1 cell focus assays

Vector	Exp. 1	Exp. 2	Number of foci [†]		Exp. 5	Exp. 9
			Exp. 3	Exp. 4		
ile12N	0, 4	3, 3, 7				
ile12T	21, 21	46, 53, 70				
trp12N			1, 1	1, 0	1, 0, 0	
trp12T			7, 6	15, 2	8, 8, 18	
	Exp. 6		Exp. 7	Exp. 8		Exp. 9
ile12N	6, 4	3, 3, 7	1, 6	20, 19		
ile12N(G)	70, 31	37, 61, 46	48, 61	78, 118		
trp12N	0, 1				1, 0, 0	
trp12N(G)	12, 9				7, 8, 10	
pT24-10Δ(A)	2, 2					8, 1, 2
pT24-10Δ	29, 41					23, 41, 43

Rat-1 cells at 10–30% confluency in 60-mm culture dishes were transfected by the calcium phosphate coprecipitation procedure⁴⁴ using 2 μg plasmid DNA and subjected 5 h later to a 45 s glycerol shock. After two days the cells were passaged into 100-mm culture dishes and subsequently fed every three days. Foci of transformed cells were counted 20 days after passage. Each experiment was performed with plasmid DNA prepared anew. In addition, duplicate plates received DNA from different isolates of the respective plasmid constructs to control for possible minor alterations occurring in the course of the construction process or subsequent propagation in *E. coli*. No discrepancies in the results obtained with such duplicate isolates were observed. Each experiment included a plate of cells transfected with an unrelated plasmid. No foci were ever observed on such control plates. [†] Numbers are given for duplicate or triplicate plates.

2,719, and confirmed that in this region the two genes differ by just the A/G change at residue 2,719. To investigate whether this substitution could indeed account for the difference in focus-forming capacity between the two types of vectors, we replaced this fragment in two of the inefficient N vectors by the corresponding fragments from the efficient T vectors. Comparison of the resulting plasmids with the parental ones in the focus assay clearly demonstrated (Table 1) that substitution of the A nucleotide at position 2,719, as found in the normal gene, by the G nucleotide present in the T24 gene dramatically increased the transforming activity of the mutant genes. Conversely, when we replaced this same fragment in the pT24-10Δ plasmid by the corresponding fragment of the trp12N vector, focus-forming activity was greatly reduced. The roughly tenfold effect mirrors that initially found between the N and T vectors. These results demonstrate that a single nucleotide within the fourth intron of the human c-Ha-ras gene plays a major role in determining the potency of activating amino acid changes. We note, however, that changing this nucleotide from an A to a G is not in itself sufficient to activate the transforming potential of the normal (gly12) cellular gene. This conclusion is based on our observation that a gly12 version of the gene analogous to the *mutT* genes described above (see Fig. 1) repeatedly failed to induce foci upon transfection of Rat-1 cells. Furthermore, whereas NIH3T3 fibroblasts can be transformed by significant overexpression of the normal gly12 gene^{14,15}, when we increased the copy number of a gly12T gene present in stably transfected cells by coamplification with a selectable marker, full transformation of Rat-1 cells could not be achieved, even at the maximally tolerable levels of the encoded protein¹⁶.

A to G mutation may be somatic change

A second mutation with biological relevance in the T24 gene could either represent a polymorphism in the human population or be the result of a somatic event. If it were a polymorphism, our data suggest that somatic activation of the c-Ha-ras gene by a mutation in the coding sequence would have more dramatic consequences in carriers of the G form of the gene than in carriers of the A form. The presence of the G(2719) version could thus contribute to a genetic predisposition to tumour development. On the other hand, if the A to G change in the T24 gene represents a somatic mutation, its effect likely played an important role in the development of the corresponding tumour. To evaluate the status of this nucleotide in the human population, we designed a protocol to analyse genomic DNAs from different individuals or cell lines for the presence of a G(2719) residue in c-Ha-ras alleles. Ha-ras intron sequences including position 2,719 were amplified *in vitro* by the polymerase chain reaction (PCR) procedure²¹ using the heat stable *Taq* polymerase (refs 22 and 23; S. Kogan, personal communication); this resulted in the appearance of a single band of the expected size upon native polyacrylamide gel electrophoresis. As neither an A nor a G residue at position 2,719 defines a restriction endonuclease recognition site (a C at this position, however, creates an *RsaI* site), we developed a modification to the procedure that permits the rapid identification of mutations at sites not recognized by restriction endonucleases. This was accomplished by altering the sequence of the reverse primer such that amplification of DNA containing a G, but not any other nucleotide, at position 2,719 would generate a recognition site for the enzyme *BstEII* (Fig. 2a). This approach was tested and validated using genomic DNA from cells containing Ha-ras genes of known sequence as shown in Fig. 2b (upper panel). We analysed 44 samples of human genomic DNA for the presence of the mutation and found that none of these was sensitive to digestion by either *BstEII* or *RsaI*, indicating that in each case the position-2,719 residue was either an A or a T, but not a G or C (see Fig. 2b for examples). These results are consistent with the suggestion that position 2,719 of the c-Ha-ras gene is not polymorphic in the human population, but rather represents

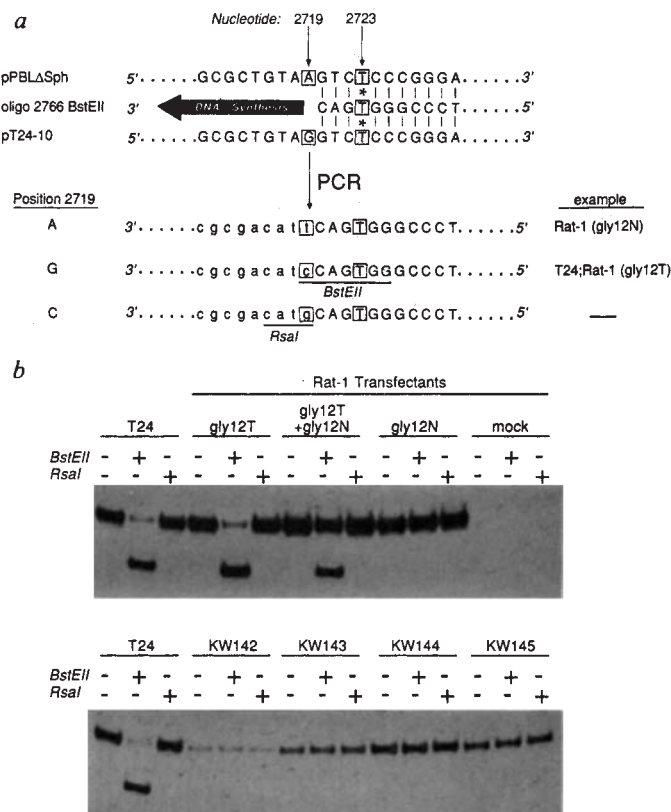


Fig. 2 Screening of c-Ha-ras genes from human individuals for the presence of a G or C residue at position 2,719. **a**, Outline of the experimental approach using a mismatched reverse primer (oligo-2766 *BstEII*) to generate a *BstEII* restriction site dependent on the presence of a G nucleotide at position 2,719. **b**, Examples of restriction enzyme analysis of PCR-amplified genomic DNA from Rat-1 cell populations stably transfected with different Ha-ras gene constructs (upper) or from lymphocytes of four healthy individuals (designated KW142 to KW145, lower). Other samples tested were six established human cell lines (including HS578T, a carcinosarcoma cell line containing an asp12-activated c-Ha-ras allele⁴⁶, and HS578Bst, established from normal cells of the same individual⁴⁷ and DNA from another 34 healthy individuals. **Methods.** Human c-H-ras sequences between nucleotides 2,633 and 2,766 were amplified essentially as described by Chehab *et al.*²², with the exception that ~0.5 pmol of 5' end-labelled forward primer (oligo 2633) was added during the 66 °C extension stage of the 29th cycle. One more round of denaturation, annealing and synthesis was completed and samples of 2 μl incubated with an excess *BstEII* or *RsaI* restriction endonuclease and electrophoresed next to 2 μl undigested material on a 10% native polyacrylamide gel, which was subsequently dried and exposed to X-ray film. Rat-1 transfectants are stable G418-resistant populations of Rat-1 cells transfected with plasmids gly12T, gly12N or an unrelated plasmid (mock) in the presence of the pSVNeoBal6 vector²⁰ specifying G418 resistance.

a site at which a somatic mutation occurred in the T24 cell line.

Although our analysis has failed to detect even a single example of the G2719 form of the gene, we should like to emphasize that it does not exclude the possibility that a G residue at position 2,719 is present at some low frequency in the human population or that other changes resulting in enhanced p21 expression occur in the region around nucleotide 2,719. Our recent results provide some substance to the latter suggestion. Thus we find that Ha-ras expression can be affected by a variety of sequence alterations in the vicinity of nucleotide 2,719 in much the same way as it is by the A to G change (unpublished observations), implying that assays in addition to the one described here will be required to identify other examples of regulatory mutations occurring in the last intron.

Mutation up-regulates expression

The mechanism underlying the difference in biological activity between mutant *Ha-ras* genes differing by just a single nucleotide in an intron is unclear. The mutation could promote a qualitative change in the protein. Its location in an intervening sequence and separate from known splicing signals would appear to limit the mechanisms by which this could be accomplished to one that would activate an alternative splicing pathway. It might, on the other hand, act to increase expression of the gene. However, there is little precedent for a point mutation acting in this way from a position within an intron distant from the gene's promoter. Nonetheless, to explore these alternatives we performed p21 immunoprecipitation analyses after metabolic labelling of cell populations transfected either transiently (human 293S embryonic kidney cells²⁴) or stably (Rat-1 cells) with several of the *Ha-ras* expression vectors described above in addition to gly12 analogues of the N and T vectors. Figure 3a shows that the p21 levels in two independent stable populations of gly12T transfected cells were well above those in gly12N transfected cells. Significantly, no qualitative differences in antibody-reactive material were apparent between the gly12N lane on the one hand and the gly12T lanes on the other, suggesting that structural differences between the products encoded by the two types of vectors play no role in the observed biological effect of the A/G alteration. To obtain a more accurate measure of the relative efficiencies of expression of the different *Ha-ras* genes, we analysed p21 levels in transiently transfected human 293S cells. Figure 3b demonstrates that such cells transfected with the 'A' form of the mutant *Ha-ras* genes expressed markedly less p21 than those transfected with the 'G' form. It thus appears that *ras* vectors containing a G residue at position 2,719 direct the synthesis of p21 much more efficiently than the corresponding vectors carrying an A. Furthermore, as in the stably transfected populations, the transient assays revealed no qualitative differences between cells transfected with the A or G forms of the gene, in accord with the notion that more efficient p21 expression by the G-forms may be the sole basis for their increased transforming activity. We also note that the results of the transient immunoprecipitation assays indicate that the effect of the A/G substitution is not limited to Rat-1 cells, suggesting that the point mutation may be of broad biological significance.

Although it appeared unlikely that a mutation in an intron could affect the amount of the encoded protein without altering messenger RNA levels, we have verified this by Northern blot analysis. As shown in Fig. 3c, the A to G change resulted in an increase in the level of steady-state *Ha-ras* mRNA, and this increase closely paralleled that seen at the protein level, confirming that the mutation acts at a stage before the formation of fully processed *Ha-ras* mRNA.

Effect on a heterologous gene

To establish whether the intron mutation described here stimulates expression by a mechanism applicable beyond the human *Ha-ras* gene, we have sought to mimic its effect in a different gene system. Based on results of exploratory experiments (data not shown) we transferred the A and G forms of a ~300-bp *NheI*-*Bst*EII fragment containing nucleotide 2,719 at a central position into a *Sma*I site in the third intron of the human growth hormone (*hGH*) gene located in expression vectors using three different promoters. The efficiency of *hGH* synthesis directed by such vectors can be quantitatively monitored on transient transfection into different cell types²⁵. Table 2 presents the results of such assays performed in human 293S embryonic kidney cells. It is evident that irrespective of the promoter used (the strong human β -actin²⁶ or cytomegalovirus major immediate early²⁷ promoters or the weak human *Ha-ras* promoter¹⁷), *hGH* expression from vectors containing the A form of the inserted *Ha-ras* fragment was approximately tenfold lower than that from the corresponding vectors containing the G form. These results are in close agreement with the data of the Rat-1 cell

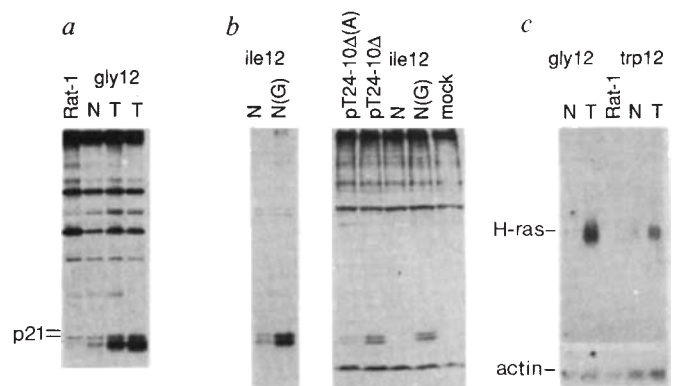


Fig. 3 Expression of c-*Ha-ras* genes in stably transfected Rat-1 cells (a, c) or transiently transfected human 293S cells (b) analysed at the protein level by immunoprecipitation (a, b) or at the RNA level by Northern blot (c).

Methods. a, Rat-1 cells were transfected as described in Table 1 with 2 μ g plasmids gly12N or gly12T in the presence of 0.1 μ g pSVNeoBal6 (ref. 20) and passaged 48 h later into selective medium containing 0.4 mg ml⁻¹ G418. After 14 days the emerging colonies (more than 50 per plate) were trypsinized and passaged en masse. Such populations were labelled with [³⁵S]methionine, lysed and subjected to immunoprecipitation using a polyclonal anti-p21 antibody⁴⁸ as described previously²⁰. The first lane shows proteins precipitated from a mass culture of G418-resistant Rat-1 cells stably transfected with an unrelated plasmid in addition to the *neo* vector. b, Human 293S cells, a suspension culture-adapted subclone of the 293 cell line²⁴, were transfected with 3 μ g *ras*-plasmid or an unrelated vector (mock) in the presence of 0.3 μ g hepatitis B virus surface antigen (HBsAg) expression vector p342E (49) and 3 μ g pUC19-AdVA containing the adenovirus-2 VA RNA genes that act to increase the translational efficiency of cotransfected genes⁵⁰. Two days later media from the cells analysed in the five lanes to the right were collected and HBsAg levels determined using a radioimmunoassay (AUSRIA II, Abbott Laboratories). Metabolic labelling and p21 immunoprecipitations were performed as above. HBsAg AUSRIA results (1:5 dilutions, in c.p.m.): pT24-10Δ(A), 16,747; pT24-10Δ, 14,640; ile12N, 4,858; ile12N(G), 9,816; mock, 15,308. c, trp12 and mock ('Rat-1') transfected populations were generated as described above for the gly12 populations except that 1 μ g vector and 10 μ g carrier DNA were used in the transfection. RNA was prepared⁵¹, electrophoresed (20 μ g) on a 1% agarose/formaldehyde gel and transferred to nitrocellulose filter paper⁴⁵. The blot was hybridized at 42 °C for 16 h in the presence of 50% formamide and 10% dextran sulphate to a nick-translated *Eco*RI-*Not*I fragment spanning the *Ha-ras* coding sequence and isolated from a cDNA plasmid⁴⁸. The final filter wash was for 30 min at 68 °C in 0.1 $\times$ SSC/0.1% SDS. After autoradiography (upper panel) the *Ha-ras* probe was removed by incubation in 0.1 $\times$ SSC/50% formamide at 65 °C for 1 h and the filter hybridized to a 5' end-labelled synthetic oligonucleotide complementary to a sequence in the 5' untranslated region of rat β -actin mRNA (lower panel).

focus-formation assays using the homologous *Ha-ras* gene, and suggest that neither a specific cell type, a specific promoter, nor any gene in particular is required for the mutation to exert its effect. The results further demonstrate that the 300-bp *Ha-ras* intron fragment contains all genetic information pertinent to the effect of the single nucleotide change. Taken together, these data strongly suggest that the mutation affects expression of the *Ha-ras* gene by a widely applicable mechanism.

Discussion

The mechanism of oncogenic activation of the human c-*Ha-ras* gene was initially defined by comparing the transforming potential of intertypic hybrids between the normal version of the gene and its oncogenic counterpart derived from the T24/EJ bladder carcinoma cell line⁵⁻⁸. Although these efforts documented that a single nucleotide change at position-12 was sufficient to activate the transforming capacity of the polypeptide, the literature

Table 2 Expression of hGH by vectors containing an Ha-ras A or G insert in human 293S cells

Vector*	hGH LEVELS†‡ (in ng ml ⁻¹)			
rasP.hGH.NB(A)	2.0, 2.3	2.8, 2.0	1.7, 1.6	
rasP.hGH.NB(G)	12.6, 17.2	36.7, 36.0	26.6, 18.4	
hAct.hGH.NB(A)	3.2, 2.4			
hAct.hGH.NB(G)	35.0, 21.6			
CIS.hGH.NB(A)	14.9, 12.2			
CIS.hGH.NB(G)	113.0, 138.1			

* Parental vector rasP.hGH contains the *hGH* gene including all five exons and the polyadenylation site between *Bam*HI and *Sph*I sites⁵² preceded by human Ha-ras sequences spanning the promoter, the first (untranslated) exon, intron A and a small section of the second exon preceding the translation initiation codon. Parental vector hAct.hGH was derived from this plasmid by replacing the Ha-ras derived sequences between *Bam*HI and *Bst*EII sites (nucleotides 1–1,280, ref. 5) by a human β -actin *Eco*RI–*Nae*I gene fragment including the β -actin promoter, the first untranslated exon and part of the first intron²⁶. Parental vector pCIS4hGH was a gift of C. Gorman. hGH expression by this plasmid is controlled by the human cytomegalovirus major immediate early promoter²⁷. An *Nhe*I–*Bst*EII ('NB') fragment from the last Ha-ras intron (nucleotides 2,563–2,852, ref. 5) containing either an A (designated NB(A)) or a G (NB(G)) at position 2,719 was introduced at the *Sma*I site in the third intron of the *hGH* gene in each of the three parental vectors. The orientation of the fragment relative to the direction of transcription is the same in these constructs as in the Ha-ras gene.

† Transient transfections of human 293S cells were performed using 3 μ g hGH plasmid and 0.1 μ g pUC.CMVcat (provided by C. Gorman) containing the chloramphenicol acetyltransferase (CAT) gene under transcriptional control of the human cytomegalovirus promoter, and in the case of the rasP.hGH vectors, 3 μ g pUC19-AdVA. Two days later media were collected and analysed for hGH by radioimmunoassay (IRMA, Hybritech). Cellular extracts were prepared and CAT activity determined⁵³. Variation in CAT activities was minor (less than 1.5-fold) and hGH numbers presented were not adjusted according to such fluctuations.

‡ rasP, hAct and CIS vectors were analysed in separate experiments and the numbers presented should not be interpreted to indicate relative promoter strengths.

provides some indications that this and other such mutations in isolation failed to recover the full oncogenic potential of the T24/EJ gene^{8,28}. We can now attribute this to the absence of a second genetic alteration responsible for increased expression of this allele.

The observation that a single nucleotide substitution within an intron can effect a tenfold increase in gene expression is to our knowledge without precedent. All mutations identified to date that act to increase expression localize elsewhere, namely to transcriptional promoter regions, and such changes rarely, if ever, realize more than a threefold increase in the level of the affected gene's product. A paradigm for this phenotype is provided by inherited disorders affecting globin gene expression (ref. 29 and refs therein). Adults with certain types of non-deletion hereditary persistence of fetal haemoglobin express levels of fetal haemoglobin that may be 10- to 20-fold higher than normal. Single nucleotide substitutions have been identified within the fetal globin gene promoters in such affected individuals, but reconstruction experiments suggest that these changes in isolation may account for but a small proportion of the total effect³⁰. It is also noteworthy that efforts to define *cis*-acting sequences required for efficient transcription by saturation mutagenesis have exposed the difficulty in obtaining up-mutants of expression: in one study only 2 of 130 randomly introduced substitutions in the promoter region of a globin gene resulted in increased expression, and this by but a factor of three³¹; all other mutations were found to be either deleterious or transparent. The results of both this and other promoter mutagenesis studies³², and the observations on the hereditary persistence of fetal haemoglobin by analogy suggest that no

single promoter mutation may be able to effect a significant increase in Ha-ras gene expression either. It is of interest in this regard that the strongest up-mutation reported in the globin study alters the CCAAT box region such that it becomes almost identical to the one found in the Ha-ras promoter. Furthermore, in our studies of the weak enhancer activity associated with a region of tandem repeats 3' of the Ha-ras gene we have found that, whereas a certain minimal number of repeats—well below that found in the human population—is required for full activity, above this minimal number enhancer strength is not affected by major variations in the number of repeats (unpublished results). Taken together with our present observations it would then appear that the c-Ha-ras promoter and enhancer elements are responsible for a basal level of expression that may be subject to extensive modulation by the regulatory element in the last intron identified here. We have as yet little insight into the mechanism by which this element functions. The A to G mutation is not located in or near sequences expected to play a role in splicing of the fourth intron, namely the 5' and 3' splice sites or the lariat branch-formation site³³. It may be noteworthy that it alters the sequence AAGT to AGGT, the latter a motif found as the core of the consensus sequence of both 5' and 3' splice sites³⁴. Interestingly, the first G of this sequence in a 3' splice site is absolutely required for proper functioning of the site³³. It is possible that the presence of such a sequence alters the efficiency of splicing of the fourth intron and in this way affects Ha-ras p21 levels. However, if this sequence functions as a new splice site, the mutation would be expected to down-regulate expression by interfering with normal Ha-ras RNA splicing, while in fact it has the opposite effect. Alternatively, it is conceivable that alternate splicing occurs on RNA transcribed from the normal gene, and that this pathway is abolished as a result of the mutation. We have addressed this possibility by Northern blot analysis of mRNA from established cell lines and from the stably transfected gly12N and gly12T cell populations described above using probes to the intron sequence around position-2,719, and have failed thus far to detect any novel mRNA (data not shown). Moreover, alternative splicing would not be expected to result in a significant reduction in overall message levels detected by our complementary DNA probe, unless additional factors play a role as well. Hence, mechanisms other than those involving the splicing pathway of the c-Ha-ras gene must be considered. For example, the single nucleotide change could modify expression efficiency if it were located within a transcription regulatory domain such as an enhancer³⁵, silencer or pausing site³⁶, or if it were to increase the stability of the Ha-ras precursor RNA. However, preliminary evidence suggests that the active intron domain, as defined by our *hGH* transfer experiments, lacks some of the hallmarks of each of the three types of transcription regulatory elements as it fails to affect expression of heterologous genes when located at a variety of positions both outside and within transcribed regions. Although we have not yet excluded an effect on the stability of the unprocessed RNA, we suspect that regulation by the intron element may be achieved by a potentially novel mechanism. Our ability to transfer the effect of the mutation to a heterologous gene system should facilitate efforts to understand this mechanism.

What are the implications of our findings with respect to the biology of *ras* oncogenes? It has been established that transformation of primary cells in culture requires the concerted action of at least two oncogenes^{37–39}. There are, however, certain conditions under which the T24 gene in isolation can affect some degree of transformation of primary cells^{40–42}. Such conditions include a level of expression tenfold above that of the endogenous Ha-ras gene, suggesting that increased expression may be functionally equivalent to some activity otherwise provided by the complementing oncogene. The recent demonstration that even a twofold reduction in the level of an activated endogenous p21 can lead to reversion of the transformed phenotype of a spontaneous human fibrosarcoma cell line⁴³

suggests that a tenfold increase in expression resulting from a mutation such as the one described here could play a decisive role in tumorigenesis.

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Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product

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One of the cellular targets implicated in the process of transformation by the adenovirus E1A proteins is a 105K cellular protein. Previously, this protein had been shown to form stable protein/protein complexes with the E1A polypeptides but its identity was unknown. Here, we demonstrate that it is the product of the retinoblastoma gene. The interaction between E1A and the retinoblastoma gene product is the first demonstration of a physical link between an oncogene and an anti-oncogene.

REGULATION of cellular proliferation is a complex process that involves both positively and negatively acting signals. Tumorigenesis results from alterations in genes whose protein products are involved in these signalling pathways. The DNA tumour viruses encode a set of proteins that are capable of overriding and reprogramming normal regulation of cellular

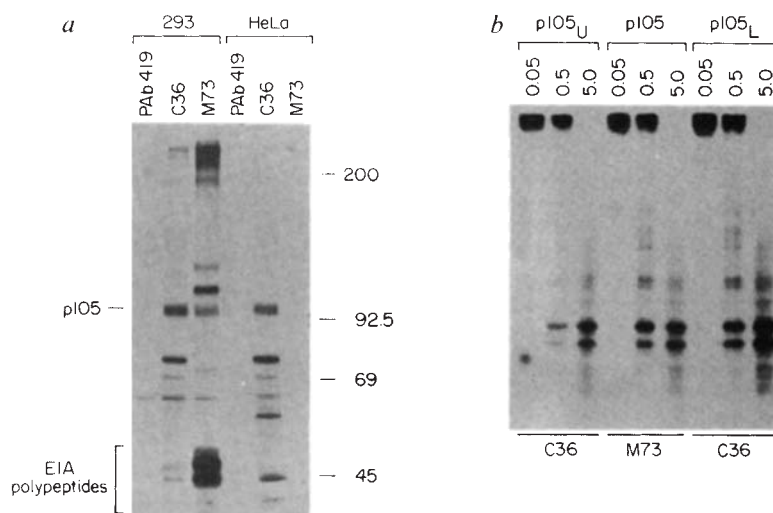
growth; consequently, they have been widely used as model systems for studying cellular transformation. The oncogenes—tumour-inducing genes—from polyomavirus, simian virus 40 (SV40) and adenovirus are able to induce a number of distinct changes in cell phenotype, including immortalization, secretion of growth factors, loss of contact inhibition, anchorage-independent growth and morphological transformation. Unlike the transforming retroviruses, these DNA viruses contain oncogenes that do not appear to have cellular homologues. Although functional similarities have been shown between cellular oncogenes

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Fig. 1 Characterization of the monoclonal antibody C36. **a**, Extracts from [35 S]methionine-labelled cultures of human 293 or HeLa cells were immunoprecipitated with M73, a monoclonal antibody specific for the E1A proteins³⁰, C36, a monoclonal antibody specific for the 105K protein, or PA6419, a control monoclonal antibody specific for SV40 large T antigen³². Proteins were resolved on a 6% polyacrylamide gel and detected by fluorography⁴⁷. **b**, Partial proteolytic maps of the 105K proteins immunoprecipitated by either C36 or M73 antibodies. Proteins from 293 cells were labelled with [35 S]methionine and immunoprecipitated using either C36 or M73. After SDS-PAGE, the autoradiogram of the gel was used as a template to excise the 105K bands. The bands that form the 105K doublet immunoprecipitated by C36 were excised and analysed separately. The upper band is labelled p105_U and the lower p105_L. Only the lower band was excised from the M73 immunoprecipitates. The proteins were subjected to partial digestion with increasing amounts of *Staphylococcus aureus* V8 protease, given in micrograms per reaction⁴⁸.

Methods. Immunoprecipitations were described previously³⁰. To prepare C36, E1A/host-protein complexes were purified from lysates of 293 cells on immunoaffinity columns prepared by cyanogen bromide coupling of M73 antibody to Sepharose 4B beads and then eluted with 1 M acetic acid. The acetic acid was removed by lyophilization and the proteins were resuspended in phosphate buffered saline (PBS). Purified proteins from $\sim 2 \times 10^8$ cells were used to immunize mice and monoclonal antibodies were prepared by fusing splenocytes to NS-1 myeloma cells⁴⁹ three days after the final boost. Positive tissue culture supernatants were identified by immunoprecipitation of [35 S]methionine-labelled extracts of 293 cells.



and those carried by DNA tumour viruses, it is not known whether these functional similarities extend to the biochemical level.

The proteins encoded by several DNA tumour-virus oncogenes form stable complexes with host-cell proto-oncogene proteins. Examples are the polyoma middle T/pp60 *c-src* complex¹, the SV40 large T/p53 complex²⁻⁴ and the adenovirus E1B/p53 complex⁵. In these cases, the formation of the complex has a profound effect either on the catalytic activity of the cellular protein, as in the case of the kinase activity of pp60 *c-src*^{6,7}, or on protein stability, as with p53^{8,9}. These changes are thought to potentiate the transforming functions of these proteins.

The mechanism of action of the adenovirus oncogene E1A is less well resolved. Acting on its own E1A can immortalize primary cells¹⁰⁻¹²; it can also cooperate with the adenovirus E1B gene or an activated *ras* gene to transform cells in culture, and these cells will induce tumours in animals^{11,13}. In addition the E1A-encoded proteins are potent regulators of gene expression, being able to modulate transcription of both viral and cellular genes (reviewed in ref. 14). The E1A proteins activate transcription of the other adenovirus early genes and certain cellular genes. They also repress transcription of genes linked to certain viral or cellular enhancers^{54,55}.

We and others have previously shown that the E1A oncoproteins associate with host-cell polypeptides known only by their relative molecular masses (M_r) of $\sim 28,000$ (28K), 40K, 50K, 60K, 80K, 90K, 105K, 107K, 130K and 300K^{15,16}. These complexes appear to mediate some of the physiological alterations induced by E1A, as any mutation that destroys binding of an E1A protein to the most predominant of the bound host proteins 300K, 107K or 105K, also inactivates the ability of the E1A oncogene to cooperate with a *ras* oncogene in transforming primary baby-rat kidney cells (ref. 17 and P.W., N. Williamson and E.H., submitted). These correlations suggest that the transforming properties of the E1A proteins depend on their binding to these host proteins.

Another class of genes involved in tumourigenesis, but apparently unrelated to either E1A or the other DNA tumour-virus oncogenes, is that of the 'tumour-suppressor' genes or 'anti-oncogenes' (reviewed in refs 18, 19). The inactivation of these genes has been implicated as a causal event in the generation of a number of different human tumours. It appears that

when both copies of an anti-oncogene are inactivated, cells initiate uncontrolled growth. Hence, the normal function of genes of this class seems to lie in blocking cell proliferation, although the molecular mechanisms are obscure.

The best studied anti-oncogene is the retinoblastoma gene (*RB* gene) inactivation of which favours the appearance of retinoblastomas and certain soft-tissue sarcomas (reviewed in refs 20-24; the abbreviation *RB* will be used for the gene and *RB* for the encoded protein, while retinoblastoma will be used to reference to the tumour). The *RB* gene exhibits large deletions in as many as 30% of the retinoblastoma tumour DNAs examined^{25,26}. Recently, we and others have isolated a full length *RB* complementary DNA²⁵⁻²⁷. The cDNA was sequenced and found to encode a protein of 928 amino acids^{27,28}. The identification of this protein as a nuclear phosphoprotein of ~ 110 K²⁹ led us to compare it with the E1A-associated 105K and 107K proteins, which are also nuclear phosphoproteins.

We report here that the 105K protein is the product of the *RB* gene, thereby demonstrating a physical and presumably functional association between the products of the E1A oncogene and the *RB* anti-oncogene. This association has unexpected implications for the mechanisms of action of these two types of gene products.

Anti-105K protein antibodies

In an initial study of the E1A proteins, a series of E1A-specific monoclonal antibodies³⁰ were prepared and used to analyse the E1A proteins in both adenovirus-infected and adenovirus-transformed cells. Immunoprecipitations of lysates from these cells yielded not only the E1A-encoded proteins, but also a series of other proteins of differing M_r s (ref. 16 and see above) that were physically associated with the E1A polypeptides. To examine these cellular proteins directly, we sought to prepare antibodies that react specifically with them. To this end, we purified the E1A/cellular protein complexes from 293 cells, a line of human embryonic cells that has been transformed by a fragment of the adenovirus 5 genome³¹, using the M73 anti-E1A monoclonal antibody, and used the complexes as immunogens for the production of further monoclonal antibodies. Among the various resulting antibodies was one, designated C36, that reacted specifically with the E1A-associated 105K host-cell protein.

The initial characterization of the C36 antibody is shown in Fig. 1a. In this experiment, lysates of [35 S]methionine-labelled

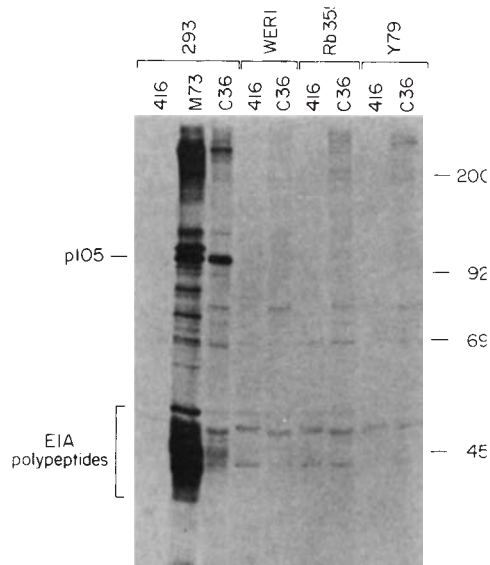


Fig. 2 Immunoprecipitations using the C36 monoclonal antibody from lysates of retinoblastoma cells. Cultures of 293, WERI-1, Y79, or RB355 cells were radiolabelled with [35 S]methionine, and lysates were precipitated with either C36, M73 or PAb416 monoclonal antibodies. PAb416 is a monoclonal antibody specific for SV40 large T antigen³². Immune complexes were collected on protein A-Sepharose beads and analysed on an 8% SDS-polyacrylamide gel by fluorography.

293 cells were incubated with antibodies C36, M73 or PAb419, a control monoclonal antibody specific for the SV40 large T antigen³². As expected, the M73 antibody precipitated the E1A polypeptides together with a group of the host polypeptides, among them the 105K protein. (The heterogeneity of the E1A proteins is due to differential splicing of the E1A primary transcript and to post-translational modifications³²⁻⁴².) The C36 antibody precipitated the 105K antigen along with a portion of the E1A proteins. The identity of the co-precipitated E1A proteins was confirmed both by immunoblotting with a panel of monoclonal antibodies that recognize several distinct epitopes on E1A, and by high-resolution two-dimensional gel electrophoresis in which the E1A proteins yield a characteristic pattern (data not shown). As shown in Fig. 1a and in other experiments below, the C36 antibody also immunoprecipitated an 85K protein. This polypeptide was recognized directly by the C36 antibodies, as it can be precipitated from lysates and partially purified preparations that do not contain the 105K protein (Fig. 2 and data not shown). As shown in Fig. 1a, several minor bands were also detected by C36 immunoprecipitation, and the reasons for the precipitation of these bands are unknown at present. Figure 1a also shows the immunoprecipitation of the 105K proteins from HeLa cells which do not contain the E1A proteins. In addition, we have found that a 105K polypeptide is specifically precipitated by the C36 antibodies (data not shown) from about 20 different common laboratory cell lines.

The 105K proteins immunoprecipitated by C36 separated into at least two bands on low percentage polyacrylamide gels. To investigate the origin of these two bands and to demonstrate that the proteins immunoprecipitated by C36 were identical to the E1A-associated 105K protein, we compared the two sets of proteins by partial proteolysis with *Staphylococcus aureus* V8 protease. As shown in Fig. 1b, both bands of the 105K doublet precipitated by C36 were similar to the 105K polypeptides precipitated in association with E1A by M73, thus demonstrating that the C36 antibodies were specific for the 105K E1A-associated protein and that both bands of the doublet were closely related.

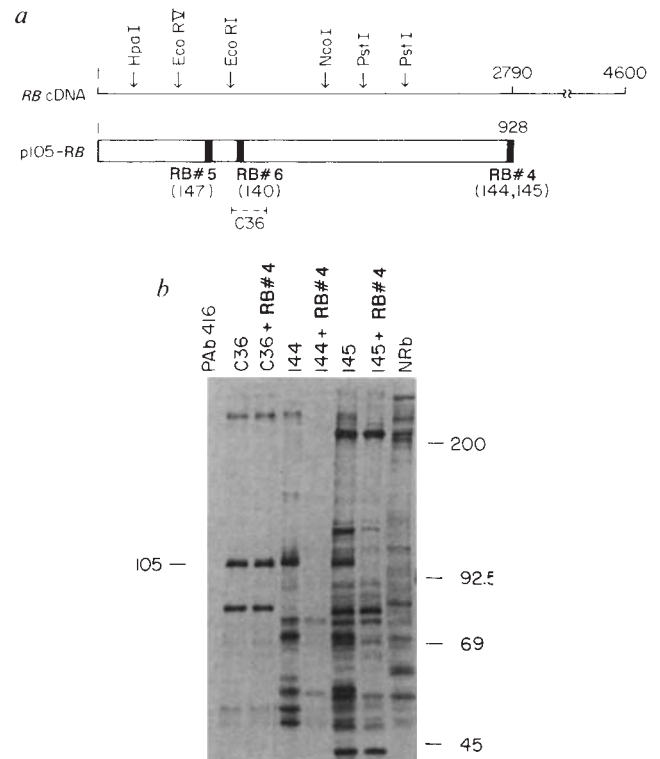


Fig. 3 Comparison of proteins immunoprecipitated with the C36 monoclonal antibody and rabbit antisera raised against peptide RB#4. **a**, Location of peptides used to produce rabbit polyclonal anti-RB antisera. Peptide RB#4 corresponds to the carboxy-terminal 15 residues of the deduced RB protein sequence (Q-K-M-N-D-S-M-D-T-S-N-K-E-E-K). Two rabbits were injected with peptide RB#4 and the sera are designated 144 and 145. The sequence of peptide RB#5 is G-S-P-R-T-P-R-R-G-Q-N-R-S-A-R and yielded serum 147. Peptide RB#6 is R-Y-E-E-I-Y-L-K-N-K-D-L-D-A-R and yielded serum 140. The predicted location of the C36 epitope is also indicated. **b**, Extracts from [35 S]methionine-labelled 293 cells were immunoprecipitated with the 144 or 145 anti-RB#4-peptide sera, the C36 monoclonal antibody specific for the E1A-associated 105K protein, or control antibodies. Immunoprecipitations were done either in the absence or presence of a saturating amount of peptide RB#4. Immunoprecipitated proteins were separated on 6% polyacrylamide gels and located by fluorography. **Methods.** Peptides were synthesized on an Applied Biosystems 430A protein synthesizer, purified by HPLC, and subjected to amino-acid analysis and mass spectrometry. In addition to the indicated residues of each peptide, an amino-terminal cysteine was included in the RB#4 peptide and carboxy-terminal cysteines were included in peptides RB#5 and RB#6 to allow convenient coupling to KLH. Coupling of the peptides to KLH was performed using N-succinimidyl bromoacetate according to Liu *et al.*⁵⁰. The peptide/KLH conjugates were injected at multiple subcutaneous sites (100 μ g peptide per injection) into male New Zealand rabbits every 2 weeks for 8 weeks.

The report from Lee *et al.*²⁹ describing the product of the *RB* gene as a 110K nuclear phosphoprotein prompted us to assay retinoblastoma cells for the 105K protein. Lysates of three cell lines containing large deletions of the *RB* gene were immunoprecipitated with C36 antibodies. The 105K proteins were not detected (Fig. 2). The similar physical properties of the RB and 105K proteins, together with the apparent absence of the 105K polypeptides from these retinoblastoma cells, suggested that the two proteins might be related.

The 105K protein is encoded by *RB*

To produce antisera that react with the RB protein, six peptides representing predicted hydrophilic regions of the RB protein

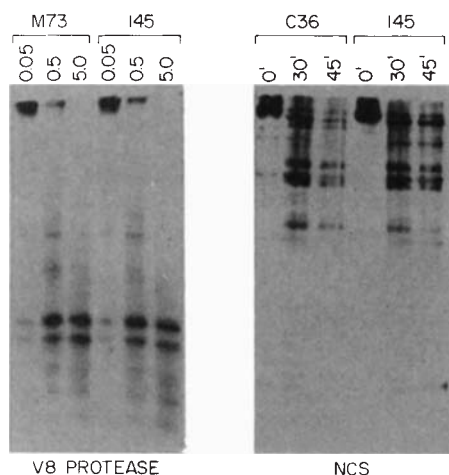


Fig. 4 Partial proteolysis of the 105K polypeptides immunoprecipitated by the 145 anti-RB#4 peptide sera and by the C36 or M73 monoclonal antibodies using *S. aureus* V8 protease⁴⁸ or N-chlorosuccinimide (NCS)⁵¹. Proteins were prepared as described in Fig. 1 and located by autoradiography. The respective bands were excised using the autoradiogram as a template. The proteins were either digested with increasing amounts of V8 protease (given in micrograms per reaction) or for increasing time with NCS, and the partially degraded products were separated on 15% SDS-polyacrylamide gels. The location of the partial proteolytic products was determined by fluorography.

were synthesized and conjugated to keyhole limpet haemocyanin (KLH). Three of these peptides, one carboxy-terminal (RB#4) and two internal (RB#5 and RB#6), induced antibodies against a protein with the properties of the *RB* gene product (see Fig. 3a for the location of peptides). Antisera against RB#4 were chosen for more detailed studies.

To compare the 105K E1A-associated protein and the *RB* gene product directly, we first measured their relative molecular masses. As shown in Fig. 3b, the 105K protein immunoprecipitated with C36 migrated similarly to RB precipitated with anti-RB#4 sera and this was true, regardless of the polyacrylamide concentration (data not shown). Immunoprecipitation by anti-RB#4 sera of the band of *M_r* 105K was inhibited by the addition of saturating amounts of peptide RB#4, but the addition of the RB#4 peptide to reactions using the C36 antibody did not block the precipitation of the 105K E1A-associated antigen. We have also compared the relative molecular masses of the 105K proteins immunoprecipitated by C36 and the RB protein recognized by an anti-trp E/RB-fusion protein antibody²⁹ (prepared and kindly supplied by Jin-Yuh Shew and Wen-Hwa Lee). Again the RB protein comigrated with the E1A-associated 105K protein (data not shown).

We next compared the polypeptides by partial proteolysis (Fig. 4). The 105K proteins were prepared either by immunoprecipitation using the M73 anti-E1A monoclonal antibody, the C36 anti-105K monoclonal antibody, or the anti-RB#4 peptide antiserum. Partial proteolysis products were prepared either by digestion with *S. aureus* V8 protease or by chemical cleavage with N-chlorosuccinimide (NCS). V8 protease cleaves after acidic residues (aspartic or glutamic acid) while NCS cleaves after tryptophan residues. Both cleavage reagents yielded identical products from all 105K proteins, showing that the positions of accessible aspartic acid, glutamic acid, and tryptophan residues were identical in these 105K polypeptides. These data prove that the 105K proteins immunoprecipitated by the C36 and anti-RB#4 antibodies were identical or closely related.

To ensure that the 105K proteins were recognized directly by the anti-peptide and C36 antibodies, and were not coprecipitated

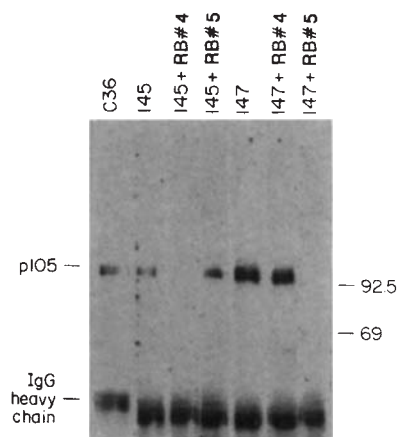


Fig. 5 Immunoblots of 105K E1A-associated protein with anti-RB antibodies. The E1A-associated 105K protein was immunoprecipitated from 293 cells using the M73 anti-E1A monoclonal antibody. The immunoprecipitated polypeptides were resolved by SDS-PAGE and then transferred to nitrocellulose membranes using standard immunoblotting techniques⁵². Strips were cut and reacted with C36, 145 (anti-RB#4), or 147 (anti-RB#5) antibodies. The binding of the anti-RB-peptide antibodies was performed with and without a saturating amount of peptide RB#4 or RB#5 and the addition of the appropriate peptide blocked the binding of these anti-peptide antibodies to 105K. After washing, the C36-reacted strips were probed with 10^6 c.p.m. of [125 I]labelled rabbit anti-mouse immunoglobulin (New England Nuclear) and the anti-RB-peptide-reacted strips were probed with 10^6 c.p.m. of [125 I]labelled goat anti-rabbit immunoglobulin antibodies (New England Nuclear). The location of the [125 I]labelled reagents was determined by autoradiography.

via another antigen, we performed two further experiments. First, as shown in Fig. 5, anti-peptide antibodies raised against two different regions of the RB protein were able to bind directly to the E1A-associated 105K protein in immunoblotting experiments. These results also confirm the specificity of the C36 antibody by demonstrating that these antibodies can also bind directly to 105K.

A complementary second set of experiments was used to test whether the C36 antibodies would specifically bind to the protein product of the *RB* gene. We synthesized RNA directly from the *RB* cDNA using an *in vitro* transcription reaction⁴³. The resulting cRNA was used as a template for translation in rabbit reticulocyte lysates. In repeated experiments, we have been unable to synthesize full-length RB protein *in vitro* using the cRNA from the human *RB* cDNA. Instead, several short products were synthesized which appear to result from initiation of translation at internal methionine residues, as their sizes (Fig. 6) agree with the predicted size of carboxy-terminal proteins beginning at internal methionines. As seen in Fig. 6, the anti-RB#4 antiserum precipitated the translation products synthesized *in vitro*, confirming its reactivity with the *RB* gene products. The anti-RB#6 and anti-RB#5 antisera, raised against internal peptides, precipitated only the polypeptides that initiate upstream of the position of their respective peptides. As expected, the C36 antibody immunoprecipitated a portion of the products of the *in vitro* translations, confirming that this antibody can bind directly to the product of the *RB* gene. These results also suggest that the epitope recognized by the C36 monoclonal antibody lies in the amino-terminal half of the protein between amino acids 300 and 380.

These last two experiments confirm that antibodies raised against either the E1A-associated 105K protein or the RB protein not only specifically recognized their respective immunogens, but also bound directly to the other protein. These data, coupled

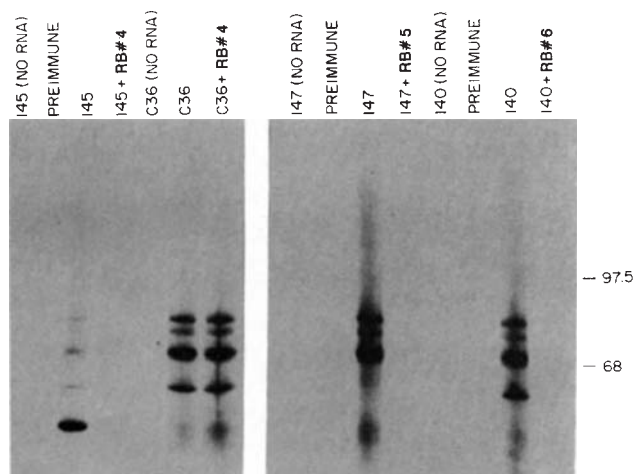


Fig. 6 Immunoprecipitation of polypeptides synthesized from the *in vitro* transcription/translation of *RB* cDNA. *RB*-related polypeptides synthesized *in vitro* were immunoprecipitated with C36, 145 (anti-*RB*#4), 147 (anti-*RB*#5) or 140 (anti-*RB*#6) antibodies in the presence or absence of saturating amounts of peptide *RB*#4, *RB*#5, or *RB*#6. Preimmune rabbit sera were used in parallel immunoprecipitations as were rabbit reticulocyte lysates without addition of *RB* cRNA.

Methods. The *RB* cDNA was cloned downstream of the T7 promoter in Bluescript pBSK⁺ plasmid (Stratagene). The plasmid was linearized by cleavage within the polylinker sequences immediately downstream of the inserted cDNA sequences and cRNAs were synthesized from the linearized templates using T7 RNA polymerase, and then translated in rabbit reticulocyte lysates (3 μ g per 35 μ l of reticulocyte lysate)^{43,53}. Immunoprecipitated proteins were resolved on 8% polyacrylamide gels and detected by fluorography.

with the biochemical data showing that the two polypeptide chains share very similar structures, lead us to conclude that these two proteins are indeed identical and are the products of the same gene. We propose to follow the nomenclature of other oncoproteins in designating this protein p105-RB.

Implications

The association of the adenovirus E1A proteins and p105-RB has important implications for the possible functions of these proteins and the genes that they represent. Perhaps the most significant of these is the connection that is now forged between these two classes of genes and their associated regulatory pathways. Oncogenes like E1A, and analogously acting cellular counterparts, allow the establishment of cells in culture ('immortalization') and collaborate with other oncogenes such as *ras* to induce full malignant transformation. The contrasting 'tumour-suppressor' or 'anti-oncogenes', represented by *RB*, have also been implicated in tumorigenesis but clearly act in a different way. Tumour formation ensues when these anti-oncogenes are inactivated, suggesting that they normally act to limit cellular proliferation. The present results show that these two classes of genes, one acting positively on cell growth, the other negatively, may directly confront one another through the interaction of their encoded proteins. Oncogenes and anti-oncogenes thus appear as constituents of a common regulatory pathway. In the absence of these results, it was plausible that oncogenes and the tumour-suppressor genes like *RB* acted as elements of two distinct regulatory pathways, each concerned with a different aspect of growth or differentiation.

The known properties of the E1A and *RB* proteins suggest a provocative model for the function of the E1A/p105-RB complex. It is known that the E1A proteins can efficiently transform cells in cooperation with an activated *ras* gene. It is also known that the disruption of both copies of the *RB* gene often leads to the appearance of retinoblastomas or other genetically related

tumours. Presumably any mechanism that interferes with the normal function of the *RB* protein would produce results similar to the loss of the *RB* gene. One possibility is that the E1A proteins inhibit the function of p105-RB by complex formation, thus achieving the same effect as the loss of the *RB* gene.

What might be the physiological role of a cellular tumour-suppressing gene like *RB*? We favour a model in which anti-oncogene proteins like p105-RB are elements of a signalling pathway that allows cells to respond to environmental signals. These signals might be differentiation inducers, cell cycle regulators, or other factors that carry an inhibitory signal to the cell. The loss of the *RB* gene and associated loss of the *RB* protein would remove an essential link in such a pathway and in this way disrupt signal transduction. As a consequence, the cell would lose its ability to respond normally to the inhibitory signal, while retaining its proliferative ability. Any resulting tumours would represent the progeny of this expanding cell population. Following this model, the E1A proteins act to block the passage of growth-inhibiting signals and in this way serve as indirect stimulators of cell proliferation.

What does the virus gain through the binding of E1A to p105-RB? For adenovirus itself, the modulation may be important for its replication. The major cell targets for adenovirus infection are epithelial cells lining the upper respiratory tract or the intestines, cells in which proliferation is inhibited or tightly regulated. One of the major goals of viral early protein expression must be to alter the physiological state of these cells and to drive them into S-phase (nuclear DNA replication). In this way, the viral oncogene creates an intracellular environment that is more permissive for viral DNA synthesis. The E1A proteins have been shown to stimulate host DNA synthesis⁴⁴⁻⁴⁶ and the virus may achieve this by antagonizing the function of cell proteins like p105-RB that normally serve to constrain cell growth within a tissue.

A growing body of literature supports the existence of genes whose actions suppress cell proliferation¹⁸. Overcoming the actions of these genes appears to be a prerequisite for achieving the tumorigenic phenotype in many cancers. The findings presented in this study suggest that one of the functions of the adenovirus E1A proteins is to overcome the action of the *RB* suppressor gene. This lends weight to the notion that cellular transformation requires not only growth stimulation, but also other equally important functions that override the mechanisms holding cell growth in check.

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LETTERS TO NATURE

Theory of adhesion for the large-scale structure of the Universe

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At present we see the large-scale distribution of matter in the Universe primarily as clusters and superclusters of galaxies, with giant voids between them^{1,2}. Understanding the origin and evolution of the large-scale structure (LSS) is one of the central problems in cosmology; it is of direct concern in understanding both the nature of the dominant dark matter in the Universe and physical processes in the very early Universe when primordial inhomogeneities were generated³⁻⁵. Here we use a new theoretical approach⁶⁻⁸ to the formation of LSS by applying the Burgers' equation⁹ that mimics the gravitational sticking of matter at the non-linear stage of gravitational instability. In this theory the non-linear evolution, including both the formation and clustering of clumps of matter separated from the Hubble expansion, is directly determined by the geometrical structure of the initial random field of linear newtonian gravitational potential fluctuations ϕ which may be gaussian or non-gaussian, depending on the model.

The potential perturbations ϕ link the present non-linear picture to the earliest stages of the Universe in a unique way, and form the basic object of our investigation, in contrast to most previous works where the distribution of the matter density ρ is studied.

Voids form around high peaks of the field ϕ , so their statistics are determined by the statistics of the peaks of ϕ . The observed sizes of voids are determined by the mean amplitude of primordial inhomogeneities $\langle \phi^2 \rangle^{1/2}$ which leads to a new method for scaling amplitude ϕ (L.A.K., preprint). Also, the falling of the nearest superclusters towards a great attractor may be interpreted as a shallow valley between high peaks of gravitational potential for corresponding space region.

The mean density of matter in the Universe is comparatively low at present, and close to the critical value $\rho_{cr} = 5 \times 10^{-30} h^2 \text{ g cm}^{-2}$ predicted by cosmological inflation (h is the Hubble constant in units of $50 \text{ km s}^{-1} \text{ Mpc}^{-1}$). On small scales, $< 100 \text{ Mpc}$, the Universe is very inhomogeneous. The largest structures, superclusters and voids, have typical sizes $\sim 30\text{--}100 h^{-1} \text{ Mpc}$, but there is also evidence of structures up to 300 Mpc^{10} in extent. We assume LSS originates by gravitational

instability of primordial inhomogeneities. Both adiabatic (inflaton) or isocurvature (isoinflaton) gaussian perturbations are inevitably generated by vacuum fluctuations during the inflationary era; non-gaussian inhomogeneities (such as cosmic strings and bubbles) may be generated by nonperturbative mechanisms, and can be also progenitors of various gravitational instability models.

In the present Universe most mass is in the form of dark, non-luminous matter, generally accepted to consist of weakly interacting particles (perhaps experimentally unknown), which are apparently of relic origin. Possible candidates are neutrinos (comprising so-called hot dark matter, HDM), or axions, gravitinos and so on (cold dark matter, CDM). At the epoch of LSS formation, dark matter is well described as collisionless, pressureless, dust-like matter. It is governed by gravitation only; the newtonian approximation suits our purposes very well^{4,5}.

The motion of dust-like matter in the expanding Universe obeys a well-known basic nonlinear system comprising a continuity equation, Euler's equation and Poisson's equation, for density $\rho(t, r)$, peculiar velocity $v(t, r)$ and gravitational potential $\phi(t, r)$ in an expanding uniform background described by a scale factor $a(t)$ and a mean density $\bar{\rho}(t)$ (refs 4, 5). The peculiar velocity is the difference between physical velocity w of an object and its Hubble velocity Hr ; $v = w - Hr$. The initial conditions are defined at the linear stage when all values v, ϕ and $(\rho - \bar{\rho})/\bar{\rho}$ are small. The usual tool to study the nonlinear evolution of the structure is N -body numerical simulation, but this approach is limited by the capabilities of present-day computers^{11,12}. Fortunately, there is the very useful Zel'dovich approximation¹³⁻¹⁴ (we assume $\bar{\rho} = \rho_{cr}$)

$$r(t, q) = a(t)(q + a(t)\nabla\Phi_0(q)) \quad (1)$$

describing the growing mode of gravitational instability in terms of particle displacements from unperturbed positions specified by lagrangian coordinates q . The initial perturbation is given by the potential vector field $\nabla\Phi_0$, which is proportional to the peculiar velocity field in the linear stage. However, approximation (1) breaks down soon after the appearance of the first nonlinear objects, 'pancakes', of supercluster size in HDM models and of cosmologically negligible sizes in CDM models.

Several essential features of 'pancake' formation and, more important, their subsequent evolution, can be simulated in an adhesion^{6-8,15}. In this model the collisionless particles move according to equation (1) until they fall into pancakes. Once there, the particles stick together, conserving momentum and moving along the pancake to form filaments, and along the filaments to form clumps. This is obviously a considerable oversimplification of the real processes in pancakes, as well as of their inner structure, but it adequately describes the global evolution of LSS beginning from some minimal scale determined

by the cut-off of the spectrum of initial (linear) density perturbations.

Here we discuss a particular model with adhesion where matter obeys an equation of motion with artificial viscosity (Burgers' equation):

$$\frac{\partial \mathbf{u}}{\partial a} + (\mathbf{u} \nabla_x) \mathbf{u} = \nu \Delta_x \mathbf{u} \quad (2)$$

where $x = r/a(t)$ is the comoving coordinate. This equation can be obtained from the basic system (see chapter II in ref. 5) using a substitution $\mathbf{u} = \mathbf{v}/a\dot{a}$, using the scale-factor $a(t)$ as the time variable and incorporating the approximate solution (1) (see ref. 8 for derivation). It is also assumed that the large-scale part of the gravitational force is reasonably well described in terms of the Zel'dovich approximation, but the action of gravity in the real system mainly results in preventing the fast growth of pancake thickness predicted by Zel'dovich. The latter effect is supposed to be mimicked by adhesion. It can be approximately described by an artificial viscosity, appearing as an additional term on the right-hand side of equation (2). The particular form of the artificial viscosity term was chosen to obtain the 3-d generalization of Burgers' equation which is well known in theory of turbulence⁹.

As we are not going to use this approximation for describing the internal structure of the pancakes it seems reasonable to make one more assumption crucially simplifying the following analysis. We will suppose that $\nu \rightarrow 0$, which means that the viscosity term works only inside the pancakes. Outside pancakes it does not influence the motion of matter and equation (2) is physically equivalent to the Zel'dovich approximation.

In contrast to vortex motions in turbulence theory, in the cosmological problem the motion is potential, with $\mathbf{u} = \nabla \Phi$. The velocity potential Φ of equation (1) can be found from the newtonian gravitational potential ϕ as $\phi = -\dot{\Psi} - (\nabla \Psi)^2/2a$, where $\Psi = \dot{a}a^2\Phi$. At the linear stage, ϕ and Φ do not change in time and are proportional to each other.

It is remarkable that the nonlinear equation (2) admits an analytical solution for potential motion⁹ (see also refs 6-8) given by $\mathbf{u} = \nabla_x \Phi$, $\Phi = -2\nu \ln U$, where

$$U = \int d^3q \exp \left(-\frac{1}{2\nu} \left(\Phi_0(\mathbf{q}) + \frac{(\mathbf{x} - \mathbf{q})^2}{2a} \right) \right). \quad (3)$$

In our simple but interesting case $\nu \rightarrow 0$, solution (3) has a very nice geometrical interpretation⁹. Let $\phi(\mathbf{q}) = -(\dot{a}a^2)\Phi_0(\mathbf{q})$ be the initial distribution of the gravitational potential perturbations in the Universe. Then at an arbitrary time $t = t(a)$ (the reverse function of $a = a(t)$) one can find the position of every particle that has not yet stuck into a pancake by means of the following procedure. Find a paraboloid $P = (\mathbf{x} - \mathbf{q})^2/2a + h$ tangential to the 3-dimensional hypersurface $\phi(\mathbf{q})$ at the point $\mathbf{q}$ by adjusting the free parameter h under the condition that the paraboloid does not cross the hypersurface at any point. One can then find the coordinate $\mathbf{x}$ of the particle in question (with lagrangian coordinate $\mathbf{q}$) at chosen time t simply by projecting the top of the paraboloid into $\mathbf{x}$ -space.

At early times (in the linear stage of the evolution of density perturbations) the scale factor $a(t)$ is relatively small and therefore the paraboloid P is sharp. It can touch every point of the hypersurface ϕ without crossing it at other points. At this stage there is one-to-one correspondence between lagrangian coordinates $\mathbf{q}$ and eulerian coordinates $\mathbf{x}$. But in the course of time $a(t)$ grows, resulting in a shallow paraboloid P that now cannot touch some points of ϕ without crossing it elsewhere. The lagrangian regions unachievable without touching have run into pancakes which are in reality the eulerian regions of multistream flows. But in our model with $\nu \rightarrow 0$ we approximate them as infinitely thin sheets. To find the eulerian position of the sheets one has to find the positions of the paraboloid where it touches the hypersurface ϕ at two points simultaneously (without crossing ϕ at other points). Again the set of the apices of the

paraboloids in these positions indicates the positions of the sheets.

At late stages one can also find the positions of the paraboloids where they touch hypersurfaces ϕ in three and even four points simultaneously. In these cases the apices of the paraboloids indicate the positions of filaments and nodes of the cellular structure.

Using this simple geometrical picture one easily comes to the conclusion that the positions of clumps of matter (which coincide with the modes at the late nonlinear stage are controlled by the highest peaks of the gravitational potential perturbations taken (and this is most important) at the linear stage.

Thus some essential aspects of the nonlinear dynamics of gravitational instability in the theory delineated above are found by scanning the 3-d hypersurface of initial perturbations of gravitational potential $\phi(\mathbf{q})$ with paraboloids of time-dependent curvature $a(t)^{-1}$. The initial inhomogeneities of the potential are defined by linear theory and are connected with perturbations of a uniform space-time metric, which at the linear stage could be written (refs 5, 16; G. V. Chirbisov and V. F. Mukhanov, preprint) in clear conformal-newtonian form

$$ds^2 = (1 + 2\phi) dt^2 - a^2(t)(1 - 2\phi)\gamma_{\alpha\beta} dx^\alpha dx^\beta \quad (4)$$

involving inhomogeneities $\phi(\mathbf{q})$ only. For the simplest case of pure adiabatic perturbations, the metric (4) obtains after inflation, and the perturbations arise due to inevitable quantum vacuum fluctuations $\delta\chi$ of the inflation-driving scalar field χ . Its amplitude $\phi_\lambda \equiv (k^3|\phi_k|^2)^{1/2} \approx (3\pi V)^{3/2}/M_{\text{pl}}^3 V(\chi)$ is determined by the parameters of the potential $V(\chi)$ of scalar field χ ; M_{pl} is the Planck mass $= 10^{19}$ GeV (ref. 16 and references therein). The spatial distribution of inhomogeneities of the gravitational potential $\phi(\mathbf{q})$ arising from vacuum fluctuations of $\delta\chi$, is described by a realization of a random gaussian field, which is characterized by statistics of its positive and negative peaks¹⁷⁻¹⁹.

In our theory with adhesion, the statistical properties of the initial 3-d hypersurface $\phi(\mathbf{q})$ are manifested in the statistics of the apices of the inserted paraboloids. Our geometrical interpretation opens the possibility of statistical investigations of the distribution of coordinates, velocities and masses of pancakes, filaments and knots (galaxies, clusters and superclusters of galaxies) already begun in refs 6-8 and L.A.K. (preprint) and L.A.K., D. Yu. Pogosyan and S. F. S. (preprint). For this we must pay, of course, by the loss of information on the inner structure of objects, and the problem of delineating LSS by its luminous baryonic matter remains beyond the scope of the theory.

Most previous papers on LSS concentrate on the distribution of the matter density $\rho(\mathbf{r})$ or the distribution of eigenvalues of the deformation tensor, which are useful both in the linear regime^{4,5} and for the 'pancake' theory^{13,14,17,20-22}. The extreme point of view within this approach connects the high peaks of the initial random field $\rho(\mathbf{r})$ with galaxies and clusters of galaxies¹⁹.

By contrast, in our approach (see also refs 6-8, 23 and L.A.K., preprint) the potential is primary, and the matter is secondary. The geometrical properties of the random gaussian (or non-gaussian) field ϕ play a crucial role. The mathematical results¹⁷⁻¹⁹ on the properties of random fields are also very useful in this approach.

At points of maximum curvature of the 3-d hypersurface, the first non-linear objects (pancakes) appear. Their statistics were investigated in refs 17, 20. The pancakes grow, some merging according to the catastrophe theory²¹, which applies here in a modified form. At the intersections of pancakes, filaments arise and in turn at the intersections of the filaments, knots form. These knots can be identified at the present epoch with clusters of galaxies. Knots can merge to form knots of larger mass. This proceeds as in the hierarchical clustering scenario²⁴ (if the initial spectrum is not too steep). The picture is confirmed by the first

numerical simulations based on insertion of paraboloids (L.A.K., D. Yu. Pogosyan and S.F.S., preprint).

The approach based on gravitational potential suggests that voids of galaxies form around high peaks of initial ϕ -field (L.A.K., preprint). As the 'wind rose' of the velocity field $\mathbf{v} \approx -\nabla\phi$ flows from a peak of ϕ , matter is swept out from its vicinity carrying away the apices of expanded paraboloids leaning against this peak. The higher the peak, the larger the void. Another argument for this origin of voids around peaks concerns the initial statistics of galaxies formation: the initial maximum of $\delta\rho/\rho \approx \Delta\phi$, or the maximum of main eigenvalue of the tensor $\nabla_i\nabla_j\phi$, connected to the first condensed objects, galaxies avoid regions of maximum gravitational potential due to their mutual anticorrelation (L.A.K., preprint; L.A.K., D. Yu. Pogosyan and S.F.S., preprint).

Thus the statistics of observed voids are determined by the statistics of the high peaks of the initial gravitational potential, which, in turn, strongly depend both on the model of dark matter and the form of the spectrum of primordial perturbations^{25,26}. The usefulness of studying voids because of their simple physics was pointed out in refs 27 and 28.

An attractive perspective would be inverse construction of high peaks of potential and its statistical properties by using paraboloids to scan observational data. We note that the gravitational potential ϕ has no high positive peaks in a standard scenario with cosmic strings.

The sizes of observed voids are connected with the amplitude of primordial perturbations of potential ϕ . A crude estimate (L.A.K., preprint) gives

$$\langle D \rangle \approx 9,200 h^{-1} \alpha \sqrt{\sigma} \text{ Mpc} \quad (5)$$

where $\alpha \approx 0(1) = \text{constant}$ and $\sigma \equiv \langle \phi^2 \rangle^{1/2}$. The value of σ is limited from above by restriction on the large-scale anisotropy in microwave background radiation $\Delta T/T \approx \sigma$ arising from the propagation of light in the inhomogeneous gravitational field ϕ (Sachs-Wolfe effect). We suggest using formula (5) together with the statistics of void sizes as a new method for scaling amplitude of initial density (rather than potential) perturbations, along with the usual scaling from the correlation function of galaxies ξ_{gg} . Formula (5) is in a reasonable agreement with observational data $\langle D \rangle \approx 50 \text{ Mpc}$ and with limitations on $\Delta T/T$. The initial inhomogeneities in the Universe and the observed LSS are uniquely connected in the theory of adhesion.

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VLBI imaging with an angular resolution of 100 microarcseconds

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The investigation of the nuclei of galaxies and quasars has been central to modern astronomy. The application of the technique of very-long-baseline interferometry (VLBI) to such sources has allowed the mapping of their angular radio brightness distributions and the analysing of their temporal changes. Many radio galaxies and quasars consist of a compact core, or centre of activity, and collimated jets of material ejected from the core with relativistic velocities¹. Of particular interest are the brightness distributions and kinematics of the cores of such sources, because they may contain a supermassive object (SMO), possibly a black hole² (but see ref. 3 for an alternative view), with a mass of $\sim 10^9 M_\odot$ (ref. 4) and a Schwarzschild radius of $\sim 3 \times 10^{14} \text{ cm}$. We present here global VLBI observations of 11 radio sources at a wavelength of 7 mm, including a map of the brightness distribution of the active galactic nucleus in 3C84. This map has an angular resolution of 100 microarcseconds, unsurpassed in an image of a celestial object.

The core is probably the most optically thick part of an active galactic nucleus. As a result of an SMO's deep gravitational potential well, matter would be accreted from an extensive environment, perhaps encompassing a major part of the entire galaxy or quasar. But only the inner $10^3 r_s$ (r_s is the Schwarzschild radius) of such an SMO is believed to account for the violent activity and power output in active galactic nuclei. In this region, the jets would be collimated⁵ and the nonthermal X-rays emitted (by the synchrotron self-Compton process⁶). For one quasar, the optical output alone was inferred, for isotropic radiation, to be $10^{48} \text{ erg s}^{-1}$ (ref. 7), equal to the Eddington luminosity for an SMO with a mass of $10^{10} M_\odot$. In this setting, an SMO is at the centre of mass of the galaxy or quasar, which presumably moves transversely with respect to the microwave background. The accretion disk of an active galactic nucleus has not yet been resolved with VLBI, and only upper limits on proper motions of quasar cores have as yet been determined (see ref. 8 for the smallest upper limit yet). But substantial improvements in the resolving power of VLBI might permit the mapping of the radio brightness distribution in the region of radius 10^2 to $10^3 r_s$ around an SMO, and enable the proper motion of a galaxy or a quasar to be measured.

There are three ways to attempt to resolve this central region with VLBI: (1) observe nearby galaxies^{9,10}; (2) use antennas in space¹¹⁻¹⁴; and (3) use short radio wavelengths^{15,16}, which provide the additional benefit of being able to probe more deeply into the optically thick cores.

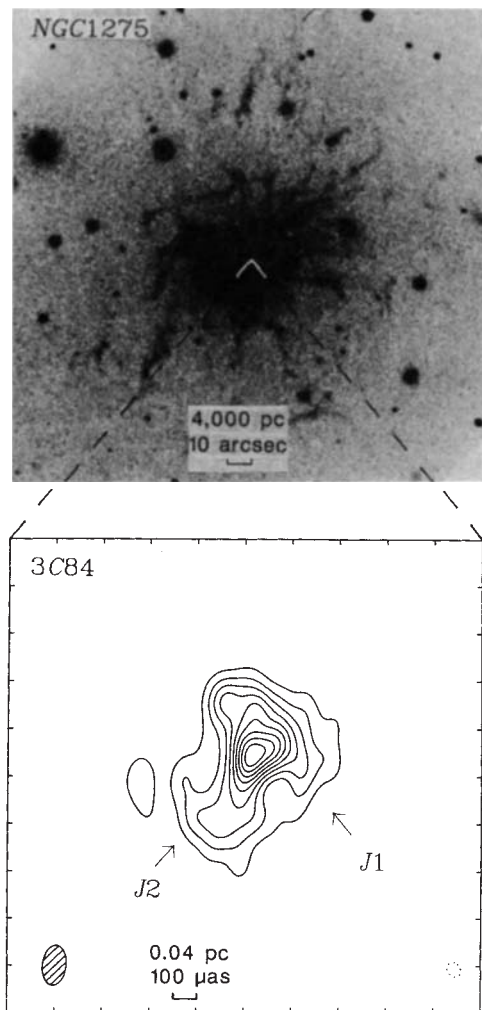


Fig. 1 A narrow band $H\alpha$ photograph²¹ of the galaxy NGC1275 and a hybrid map of the galaxy's nuclear region at 7 mm. North is up and east to the left. The total flux density in the mapped region is 6.9 Jy. The contours are at $-10, 10, 20, 30, 40, 50, 60, 70, 80$ and 90% of the peak brightness of 1.2 Jy per beam area, equivalent to $\sim 5 \times 10^{10}$ K. The 50% contour of the restoring beam with a FWHM size of $100 \times 170 \mu\text{as}$ and a position angle of -7° is shown as the striped ellipse in the lower left corner. The tick marks are separated by $200 \mu\text{as}$.

Using the Mark III VLBI system in recording mode A (ref. 17), we observed the radio galaxy 0316+413 (NGC1275, 3C84) and ten other active galactic nuclei at 7 mm wavelength (43.123 GHz), with an array of six antennas in a session on 9–10 May 1986. This session was the last and most successful in a series of nine that started in 1982, in a programme begun ten years ago by one of us (I.I.S.) and K. I. Kellermann. Preliminary results from these observations were given earlier^{18,19}.

The observations in May 1986 were correlated with the Mark III processor of the Max-Planck-Institut für Radioastronomie. After correlation, we searched for fringes in each of the ~ 750 s long 'scans.' When fringes were detected, we used the resultant delays and delay rates to roughly double our sensitivity for those corresponding scans that had not, at first, yielded detections: we narrowed the search windows approximately tenfold or, in cases where closure observables could be calculated, even to single points. We then segmented all data into intervals of 20 s to both optimize the signal-to-noise ratio and minimize coherence losses²⁰. After determining the delay rates for each segment

as a running mean over five segments, we averaged the data incoherently over 5-min intervals and calibrated the amplitudes to yield correlated flux densities.

We chose 3C84 as a prime candidate for mapping with VLBI, because it is the strongest known compact source in the northern sky at a wavelength of 7 mm. The source is associated with the Seyfert-like galaxy NGC1275 (upper part of Fig. 1), the brightest galaxy in the Perseus cluster. Its redshift of $z = 0.018$, and a Hubble constant of $H_0 = 65 \text{ km s}^{-1} \text{ Mpc}^{-1}$, imply the galaxy is at a distance of 80 Mpc. The source exhibits variability at X-rays²², optical²³, infrared²⁴ and radio^{25,26} wavelengths, reaching a factor two over a time interval of weeks, for example, at 3 mm²⁵. The structure of this galaxy and its radio source are unusually complex, displaying, in the $H\alpha$ band, filaments that emanate from the inner parts of the galaxy²¹ and showing, at a radio wavelength of 6 cm, a strongly emitting nuclear region, with an inverted spectrum, that radiates $\sim 99\%$ of the total flux density from a region not larger than a few arcseconds. This region is straddled by northerly and southerly located components that are embedded in a more extended emission region of ~ 30 arcsec (12 kpc) in extent^{27,28}. The nuclear region itself contains a compact, but complex, emission region of order 1 mas (0.4 pc) in extent, from which a ~ 10 mas (4 pc) long jet emanates in a southerly direction with a speed of $\sim 0.7c$ (refs 16, 29–31).

To produce an image of this ~ 1 mas emission region from our correlated flux densities and closure phases for 3C84, we started from a model of the brightness distribution consisting of a 'core,' two components hereafter called J1 and J2, and a halo, with their estimated parameter values given in Table 1. We then used the Caltech hybrid mapping package and produced the map shown in the lower part of Fig. 1. The correlated flux densities, self-calibrated with a constant gain correction factor for each station, the corresponding closure phases, the predictions from the map, and the U-V coverage are shown in Fig. 2. The consistency of our measurements, and hence the reliability of our calibration procedure, is demonstrated in Fig. 2 by comparison of data obtained on 9 May with data obtained on 10 May 1986.

To search for any changes of the structure of the nucleus of 3C84, we reanalysed data obtained with the B-K and T-K interferometers (see Fig. 2) in February 1985 (ref. 19). We then used weighted-least-squares to estimate the position angles of components, representing J1 and J2, of a model of two elliptical gaussians. We obtained angles of $40^\circ \pm 10^\circ$ and $135^\circ \pm 10^\circ$ respectively, not significantly different from the equivalent angles of the May 1986 components.

Models made from observations at 1.3 cm in April 1981 (ref. 30) and at 3.4 mm in March 1985 (ref. 16) reveal a dominant elongated component with a position angle of $40^\circ \pm 20^\circ$ and $15^\circ \pm 10^\circ$ respectively. Both values are consistent to within two standard deviations with the position angle of $40^\circ \pm 10^\circ$ of our component J1, and apparently indicate that J1 has existed for a minimum of several years over a wavelength range from at least ~ 1.3 cm to ~ 3.4 mm. Only the 1.3 cm model suggests the existence of a component resembling J2. But as that component's position angle is $105^\circ \pm 5^\circ$, significantly different from J2's, it is not clear whether both components are physically the same.

Taking the 3.4 mm model, and assuming that no significant flux density changes occurred between the epochs of the 3.4- and 7-mm observations, we derive a corresponding spectral index, $\alpha (S \propto \lambda^{-\alpha})$, for the core of $\alpha = -0.4 \pm 0.5$, for J1 of $\alpha = -0.6 \pm 0.4$, and for the total source of $\alpha = -0.35 \pm 0.30$. If we assume that the core and the components are synchrotron-self-absorbed above a wavelength of ~ 1.5 cm, then the magnetic field in the core is ~ 0.02 G and in the components ~ 1 G. The lifetimes of electrons radiating in such fields are a few years and a few months respectively, which is consistent with the electrons not having to be reaccelerated either over the extent of the core or over the lengths of the components. The model parameters given in Table 1 imply a brightness temperature for

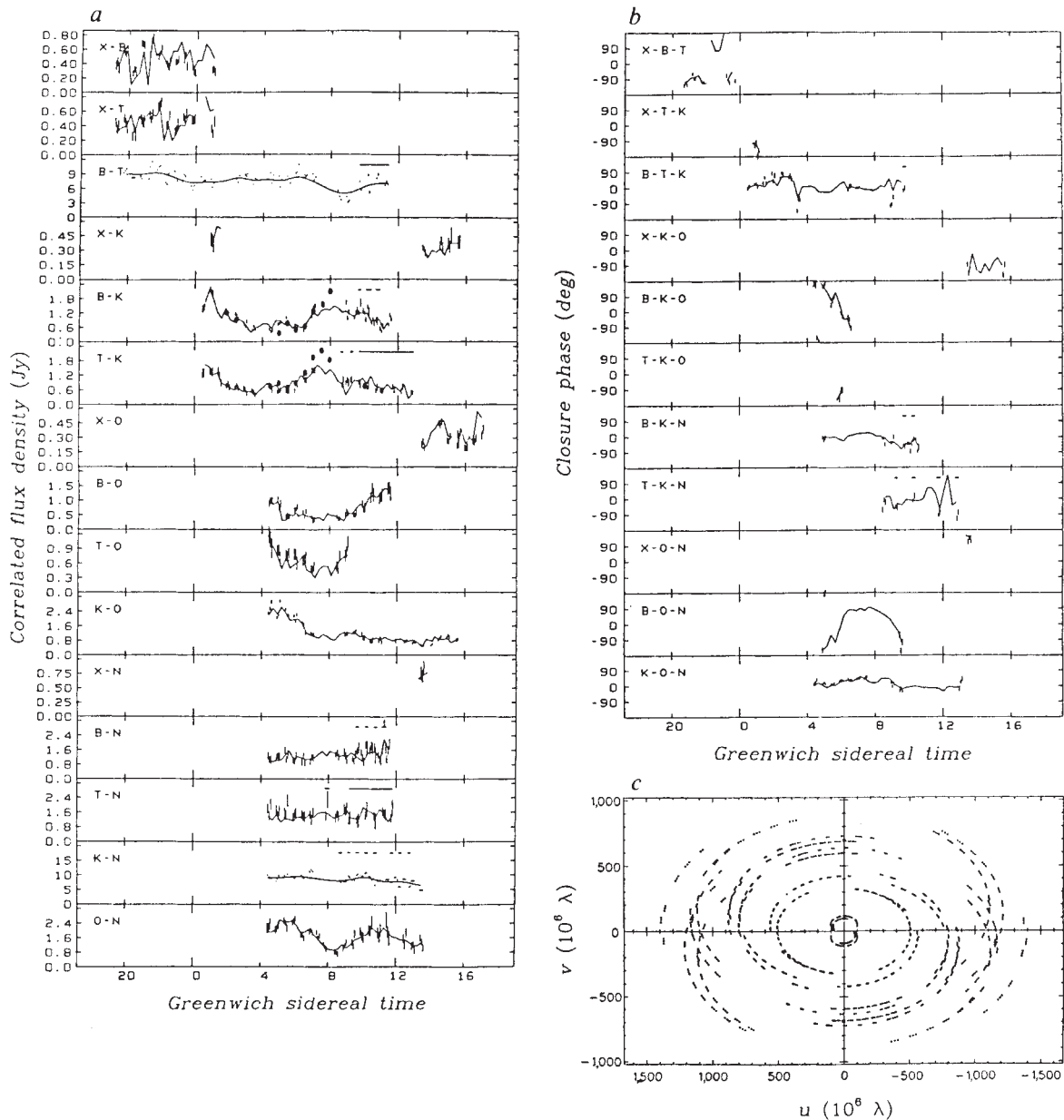


Fig. 2 Correlated flux densities (a) and closure phases (b) shown with $\pm 1\sigma$ statistical standard errors, and the corresponding U-V coverage (c) for 3C84. The solid lines in (a) and (b) represent the predictions from the CLEAN components used to produce the hybrid map shown in Fig. 1. The antennas used with their diameters, peak aperture efficiency, peak sensitivity, receiver type, system noise temperature and affiliation are: B: 100 m, 13%, 0.370 K Jy $^{-1}$, cooled mixer, 600 K, Max-Planck-Institut für Radioastronomie; X: 45 m, 53%, 0.306 K Jy $^{-1}$, cooled mixer, 530 K, Nobeyama Radio Observatory; T: 20 m, 47%, 0.056 K Jy $^{-1}$, SIS mixer, 420 K, Onsala Space Observatory; K: 37 m, 10%, 0.039 K Jy $^{-1}$, maser, 120 K, Haystack Observatory; O: 40 m, 7%, 0.032 K Jy $^{-1}$, maser, 160 K, Owens Valley Radio Observatory; N: 26 m, 15%, 0.029 K Jy $^{-1}$, uncooled mixer, 630 K, Naval Research Laboratory. Each of the antennas received left circular polarization, except for B, which received linear polarization and therefore had an effective sensitivity for VLBI observations half as large as given above. At each station, a hydrogen maser frequency standard was used. Significant correlation losses occurred at B due to phase fluctuations caused by power-line modulations of 50 Hz, and at T due to other modulations at lower frequencies. (The corresponding amplitudes were, however, corrected for these losses.) Other losses for each of the interferometers are estimated cumulatively to be smaller than $\sim 15\%$. Closure phases for which there were any two component segments of a scan that differed from each other by more than 50° or 4σ were assumed to be caused by structure outside our mapping area and were therefore deleted. Observations made on 9 May 1986 are marked by horizontal bars. Unmarked data points were obtained from observations on the following day. For the B-K and T-K interferometers, we also show the correlated flux densities (filled ellipses) for 3C84 with an arbitrary offset at epoch 25 February 1985.

the core of $\sim 10^{11}$ K and a value about fivefold smaller for each of the components J1 and J2. These characteristics, along with the stability of the position angles of the components, are similar to those for many core-jet VLBI sources. But there is one unique feature: the roughly perpendicular orientation of the two jet components, which has not been observed in any other source.

What is the nature of the core and of these two almost perpendicularly oriented components in the nuclear region of 3C84? Does each of the components perhaps represent the more compact and optically thicker inner parts of the ~ 10 mas long, southerly oriented jet? Or does each belong to the perhaps more complex region, associated with the jet that may be emanating

Table 1 Model parameters for 3C84

Gaussian component	Flux density (Jy)	Separation of centre from an arbitrary Origin (mas)	Position angle of line from origin to component (deg)	FWHM of major axis (mas)	FWHM of minor axis (mas)	Position angle of major axis (deg)
Core	1.0 ± 0.2	0	0	0.12	0.04	50
J1	2.1 ± 0.3	0.1	-80	0.6	0.06	$40 \pm 10^*$
J2	0.9 ± 0.2	0	0	0.5	0.05	$140 \pm 10^*$
Halo	8 ± 2	0	0	0.9	0.7	120
Unseen	33 ± 2					
Total	45 ± 2					

The zero values indicate parameters held fixed at the origin. The uncertainties are intended to represent one standard deviation and are omitted if they are irrelevant for the discussion. For the uncertainty of the total flux density, see Table 2. Estimates of other uncertainties are based on an inspection of the results of various model fits and on educated guesses.

* The position angles were determined from B-K and T-K data only, to allow a more accurate comparison with the corresponding position angles of the 1985 data.

Table 2 Compact radio sources

Source	Other name	Optical identification	Total flux density at $\lambda 7$ mm* (Jy)	Length of long projected baseline† (10 ⁹ λ)	Corresponding maximum correlated flux density‡ (Jy)	Corresponding maximum visibility
0133+476	—	quasar	2.5 ± 0.3 §	0.7 (TK)	0.72 ± 0.15	0.29
0234+285¶	—	quasar	2.2 ± 0.3	0.7 (BK)	detected	—
0316+413	3C84	galaxy	45 ± 2	1.4 (XK)	0.48 ± 0.09	0.01
0355+508	NRAO150	empty field	4.0 ± 0.4 §	1.2 (BX)	1.20 ± 0.10	0.30
0851+203	OJ287	BL Lac	10 ± 2	1.2 (BX)	0.85 ± 0.07	0.09
1226+023	3C273	quasar	15 ± 1	1.3 (BO)	0.57 ± 0.12	0.04
1308+326	—	BL Lac	2.5 ± 0.5 §	1.2 (BO)	0.50 ± 0.07	0.20
1641+399	3C345	quasar	9.4 ± 0.5	0.8 (BK)	0.85 ± 0.10	0.09
1739+522¶	—	quasar	1.0 ± 0.2	0.7 (BK)	not detected	—
1803+784	—	BL Lac	1.7 ± 0.3	1.2 (BX)	0.35 ± 0.06	0.21
2251+159	3C454.3	quasar	6.9 ± 0.3	1.2 (BX)	0.41 ± 0.06	0.06

* The uncertainties are derived from a combination of the calibration uncertainty and of the scatter of individual measurements and are intended to represent one standard deviation.

† Entries are of projected baseline, for the interferometers with long baselines.

‡ Statistical standard errors only are listed to indicate the signal-to-noise ratio. Calibration uncertainties are ~15%.

§ The flux densities for these sources were derived from an interpolation of values obtained from observations at several epochs between February and June 1986 and at several wavelengths (21, 8 and 3.4 mm), with each set of epochs and wavelengths straddling our observing epoch and wavelength (H. D. Aller and M. F. Aller, W. M. Kinzel and W. A. Dent, and H. Terasanta, personal communications). The other flux densities were determined at B.

|| The listed values could be larger as none of the sources other than 3C84 at stations K and O was strong enough to be detected between scans and could therefore not be used for pointing corrections during the observations.

¶ The sources were observed on 25–26 February 1985, all other sources on 9–10 May 1986.

normal to the accretion disk surrounding the hypothesized (rotating) black hole?

As to the first possibility, we find it intriguing to compare our map in Fig. 1 with the prediction from a model of a magnetized rotating SMO (ref. 5). According to this model, charged particles emanate through the sides of the light cylinder of a rotating SMO and are focused into a jet and a counterjet of particles at a distance from the SMO $\sim 10^{17}$ cm. Our component J2 and the southwesterly part of component J1, both only a few times 10^{17} cm long, straddle the southerly oriented jets which are of different lengths and known from previous observations. The northeasterly directed part of component J1 could then be part of the jet that is focused into a northerly direction. As to the second possibility, we find the morphology revealed in our map tantalizing because it is suggestive of an accretion disk (J1 or J2) around a centre of activity (core) from which a jet (J2 or, respectively, J1) emanates. But we emphasize the very speculative character of our suggestions and stress the need for further and more accurate observations at millimetre wavelengths. Also needed is a more detailed (theoretical) model of the evolution

of the core and jets before any reliable conclusions can be drawn about the nature of the source.

Apart from 3C84, we observed ten more extragalactic sources (Table 2) in 1985 and 1986, most of them selected from a survey of 26 sources at 1.3 cm (ref. 32). All but one were sufficiently compact and strong to be detected and probably mapped with our 7-mm VLBI array in future experiments.

The successful imaging of a celestial source with an angular resolution of 100 μ as at a wavelength as short as 7 mm, and the detection at this wavelength of other (including superluminal) compact sources suitable for high resolution imaging, indicate a promising future for millimetre-VLBI. More than four years were necessary to improve the performance of the array to its current status. Further improvements are possible, for example in higher local oscillator purity at B and T (see Fig. 2 legend), and perhaps at other stations. Indeed, such higher purity has already been achieved in our follow-up observations (June 1987, V.D. *et al.*, manuscript in preparation). Implementation of a circularly polarized feed at B (see Fig. 2 caption) would improve the sensitivity of all interferometers with B as one element by

~40%. Other improvements could include recording a twofold larger bandwidth at all stations, a twofold increase of the aperture efficiency of K from a rerigging of the antenna, an improvement in the thermal control of the panels, and more accurate pointing at K, and perhaps also at N and O. In the longer term, water vapour radiometers at each station could be used to correct for changes in the excess path length and in fringe phase drift arising from atmospheric water vapour³³. Other millimetre-wavelength telescopes (such as IRAM's 30-m instrument on Pico Veleta, Spain, which is the largest among about ten suitable telescopes) not yet used for millimetre-VLBI could improve the sensitivity of the array. Finally, the Very-Long-Baseline Array³⁴ with ten antennas, each usable at wavelengths down to ~3 mm and equipped with low noise receivers and a recording capability of up to 256 MHz, will eventually help define a new standard for imaging celestial sources and for investigating their kinematics at millimetre wavelengths.

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The light echoes from SN1987A

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Direct imaging^{1,2} of supernova 1987A has revealed two semi-circular arcs around the explosion site. Similar arcs around Nova Persei^{3,4} and Nova V732 Sagittarii⁵ have been explained as light echoes from the time of maximum luminosity of the outburst. Here we report the results of deep imaging and spectrophotometry of the arcs around SN1987A. The spectrophotometry verifies the nebular arcs as echoes of the light of SN1987A near maximum. The clouds must be at distances of 115 and 305 pc in front of SN1987A. We also observe an apparent proper motion for the inner echo of 1.8 arcsec per month which, at 16 times the speed of light, is highly superluminal.

Direct images of SN1987A were obtained⁶ on 19–21 March 1988 UT at the $f/7.5$ focus of the Cerro Tololo Interamerican Observatory (CTIO) 1.5-m telescope with charged-coupled devices (CCD) and UBVRI filters. The arcs were clearly seen in all colours, but the R frames provided the clearest contrast against the underlying nebulosity of the complex emission regions surrounding 30 Doradus. A montage of these frames is presented in Fig. 1. Two very obvious semi-circular arcs are

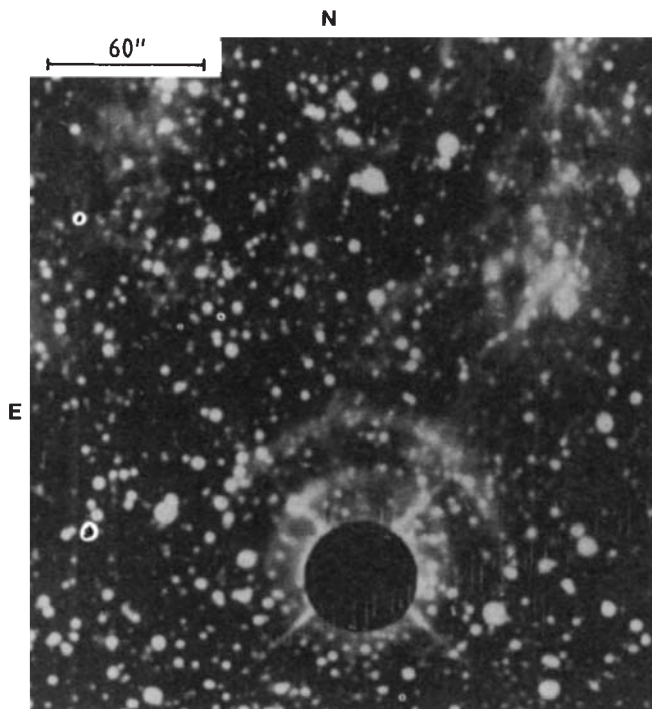


Fig. 1 Montage of five R-band CCD frames of the light echoes near SN1987A from data taken at the CTIO 1.5-m telescope on the nights of 19–21 March 1988. Each frame was a 30-min exposure with a TI CCD. The overexposed region around SN1987A has been masked with a black circle, and the four spikes emanating from the circle are diffraction images from the telescope's secondary mirror support. Note that the black circle is not centred on SN1987A, but that the spikes are. To the north two arcs with radii of 33 and 54 arcsec are visible. These are light echoes from the period of maximum luminosity of SN1987A. The very large HII region complex of 30 Doradus is 20 arcmin northeast of SN1987A.

present towards the north, in the direction of pre-outburst nebulosity.

The inner and outer arcs have radii of 32.8 and 54.0 ± 0.5 arcsec and cover about the same position angle with respect to SN1987A. The centres of curvature are coincident with SN1987A to within 2 arcsec. Neither arc is a complete ring; the outer arc extends from position angle (pa) -90° , through 0° to $+60^\circ$ and the inner arc from pa -75° to $+50^\circ$. A small arc of radius 52 arcsec is visible at pa -40° . A simple division of different colour frames showed that the arcs have quite uniform colours. For three star-free regions in the northern part of the outer arc, we find from the average colour over 20 square arcsec along the arc that the surface brightness is $V = 21.28 \pm 0.04$ magnitudes per square arcsec, and the colours are $B - V = 1.16 \pm 0.02$, and $V - R = 0.35$. We have derived these colours from photometric standards and local sky close to the arc region to account for the strong HII region contamination. The $V - R$ colours varied from 0.26 to 0.51 in the outer arc; this may result from changes in the strength of the HII region emission lines. The inner arc at pa $+40^\circ$ had a surface brightness of $V = 20.84$ magnitude per square arcsec, and $B - V = 1.20$. At a fainter part of the inner arc, we find $V = 22.75$, $B - V = 1.10$, and $V - R = 0.40$. These colours are quite different from the March 1988 colours of SN1987A, which are $B - V = 0.96$ and $V - R = 1.11$. The region between the inner and outer northern arcs is slightly brighter than that just outside the outer arc. Inside the inner arc there may also be enhanced brightness, but we cannot rule out scattered light from the seventh magnitude supernova. Rosa *et al.*² reported that the arcs are complete rings, but a published image⁷ shows that any southern extension of the outer arc is very faint. We find that the southern half of the arcs must be fainter than 24 magnitude per square arcsec.

Figure 2 compares the brightest knot in the inner arc at pa $+40^\circ$ with a CCD frame taken on 27 January 1988 with the CTIO 0.9-m telescope. The knot has clearly moved outwards by 3.0 ± 0.5 arcsec, which implies a rate of 1.8 arcsec/month. One light year at the distance of the Large Magellanic Cloud (LMC) is 1.3 arcsec, so the apparent motion is highly superluminal, at $\sim 16c$. The relative intensity along the arc in Fig. 2 has also changed.

Spectra of the rings reproducing the spectral features of SN1987A would be definitive proof that they are indeed light echoes and would allow us to estimate when the observed light was emitted. On 19.13 and 20.10 March 1988 UT, spectra from 3,750 to 5,250 Å, with a resolution of 8 Å, were obtained of the brightest knots in the outer and inner rings with the RC spectrograph and Air Schmidt GEC CCD system on the CTIO 0.9-m telescope. Figure 3 shows these two spectra, with a spectrum of the supernova taken on the same night.

Using the CTIO/Yale 1-m telescope two-dimensional photon-counting detector, we have obtained spectrophotometry⁸ of the supernova almost nightly until the end of June 1987 and more or less weekly since then, at similar resolution to the present observations. To form a sequence of comparison spectra to allow age dating of the echo, we binned the archival data by co-adding spectra in one-month intervals. In addition, an average spectrum at maximum light was constructed. SN1987A rose to maximum slowly, and faded rapidly⁹; from 4 April to maximum (22 May), it brightened by a factor of two, and then faded by a factor of two by 14 June, giving a FWHM (full-width, half-maximum) of 71 days. During this time, SN1987A emitted 60% of the total luminous energy emitted over the first 200 days. We co-added the spectra from this 71-day period to produce an average maximum-light spectrum. Inclusion of the preceding or following months did not change the average spectrum significantly.

Figure 3 shows the average spectra for March, April and May 1987, the mean spectrum at maximum light and the present spectra of the echoes and SN1987A. The spectra of the echoes are clearly different from the present supernova spectrum; note the presence of the strong H β profile in the present SN1987A

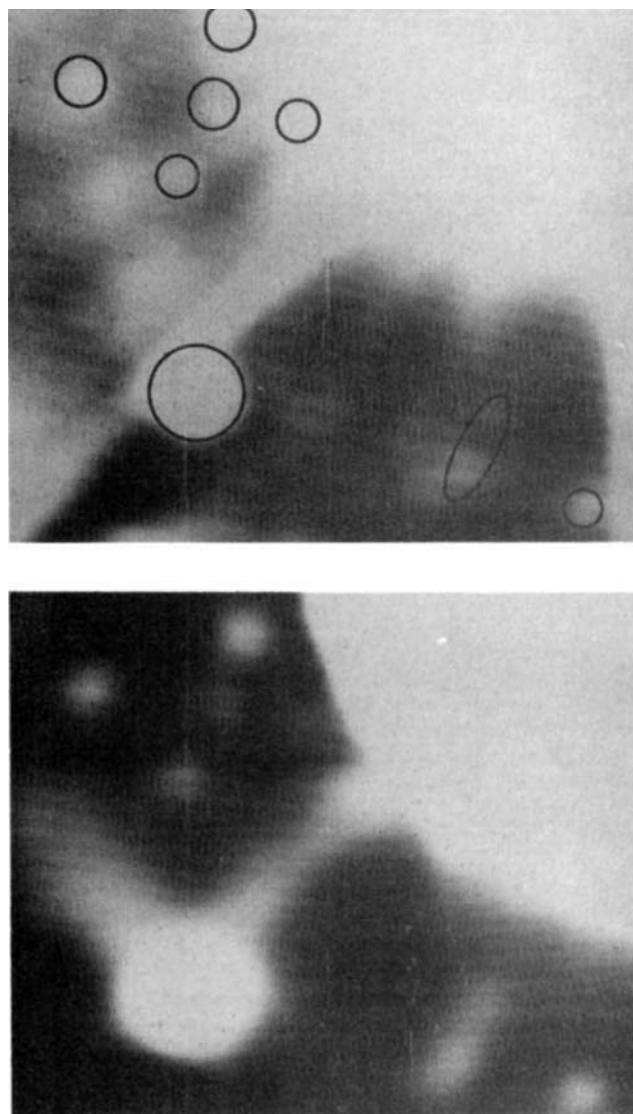


Fig. 2 Closeup of the inner echo at position angle 45° , showing the proper motion of the echo. The upper panel shows the echo on 27 January 1988 and the lower panel on 20 March 1988. The obvious stars and pre-SN1987A features have been marked in the upper panel.

spectrum and its absence from the echoes. Compare also the strengths of the H β feature in the archival data. In March and April, broad hydrogen nebular emission lines appeared on both sides of the P-Cygni emission peaks of the Balmer series. The blueward emission of these satellite lines tended to fill in the absorption in the P-Cygni Balmer line profiles¹⁰⁻¹². In April, the absorption component was almost completely gone, while in May and after, it rapidly strengthened again. Neither echo spectrum resembles the mean March 1987 spectrum in the H β region, but the spectrum of the outer echo is similar to the April spectrum of SN1987A. The spectrum of the inner echo appears to be intermediate between the April and May spectra, and is consistent with the mean spectrum at maximum light. Thus, the light that we see as the outer echo travelled 11 light months further than what we see directly from SN1987A, and the inner echo about 10.5 light months further. This spectrophotometry shows unambiguously that the arcs are indeed light echoes from SN1987A near maximum light.

The geometry and theory of light echoes has been discussed extensively¹³⁻¹⁷. The surface of reflection for a distant light echo

from a single pulse of light is approximately a paraboloid, with the source at the focus¹³. Any plane intersecting this paraboloid will be seen in projection on to the sky as a circle with a centre offset from the focus. Thus planar sheets of gas in front of the supernova will always form circular arcs in reflection. In particular, Couderc¹³ finds that (setting $c=1$) $R = (2tA + t^2(1+m^2))^{1/2}$ where R is the radius of curvature of the arc, t is the time delay between the arrival of the light pulse from maximum light and the observation of the echo, m is $\tan i$ where i is the inclination of the sheet of gas with respect to the sky, and A is the distance along the line of sight between SN1987A and the sheet of gas. The centre of curvature is offset into the direction of inclination of the sheet of gas by $mt = 1.16$ m arcsec for SN1987A. As the centre of curvature is within 2 arcsec of SN1987A, any inclination must be less than 60° for a planar sheet of gas.

Because $A \gg t$, and assuming a true distance modulus to the LMC (ref. 9) of 18.5, we find $A(\text{pc}) = 0.0963 \theta^2 (\text{arcsec})/t(\text{yr})$ where (θ) is the angular distance from the source to the echo. The expected apparent rate of expansion on the sky is 1.5 arcsec per month for the outer arc, close to the observed value. But it is possible that the observed expansion has nothing to do with the expanding paraboloid illuminating a sheet of gas. If the dust cloud is filamentary, like the reflection nebula near the Pleiades, then physically unconnected filaments can light up as the parabolic surface cuts through the cloud, in analogy to the 'Christmas tree light model'.

The echoes at 32.8, 52 and 54 arcsec must be due to clouds at 115, 295 and 305 pc from SN1987A for the time delays determined from the spectrophotometry. The fact that these are thin echoes rather than a diffuse halo around SN1987A indicates clumpiness in the gas in front of SN1987A, which is also seen in the interstellar lines^{18,19}. There are two very strong interstellar lines at the velocity of the LMC (220 and 280 km s⁻¹) which may arise from the clouds seen here. The echoes are being formed mainly in clouds to the northeast of SN1987A. This is a complex region of HII emission that appears to be associated with 30 Doradus. If this connection is not just a projection effect, it implies that SN1987A may be somewhat behind the gas in 30 Dor. Such a large line-of-sight depth to the LMC is not unexpected. Although the LMC is known to be a flattened, disk-like system^{20,21}, the scale height for its young stellar population is estimated to be ~ 400 pc (ref. 20). This is true of Magellanic-type irregular galaxies in general, in which the disk component is 'fluffy' compared to Sd galaxies²¹.

The illuminator of the clouds poses an interesting problem. We know that the two echoes are at least 100 pc apart, with little scattering material between them. The outer echo must be 26 pc in diameter, the inner echo 16 pc, conforming to our model of a sheet of gas. Yet both clouds, which are well separated along the line of sight, are brightly illuminated only towards the north, implying a high order of angular coherence to the illumination. Was something near the supernova to the south blocking the light? The so-called 'mystery-spot'^{22,23} was at pa 196°, which is the direction of the missing light. If the mystery spot was due to an occulting and reflection mechanism covering a large solid angle near SN1987A, the echoes would also be weak in that direction. Despite various attempts²⁴⁻²⁶, no convincing model for the 'mystery spot' has been generally accepted.

For a light pulse of duration $\delta(t) = 71$ days, the apparent thickness of the echo seen on the sky will be $\delta\theta \sim \theta/2t \delta t$ which for the 33- and 54-arcsec echoes implies a thickness of 3.5 and 6 arcsec. From our data, we can only roughly estimate the thickness at about 5–10 arcsec.

Because the spectrograph slit was smaller than the apparent size of the echo, we are sampling only a small part of the light pulse with the slit in the model of a planar sheet of gas. In any echo, the light at a larger radius will come from light which left SN1987A earlier, but the thickness of the gas sheet must be taken into account. From equation (2) in ref. 15, the path length

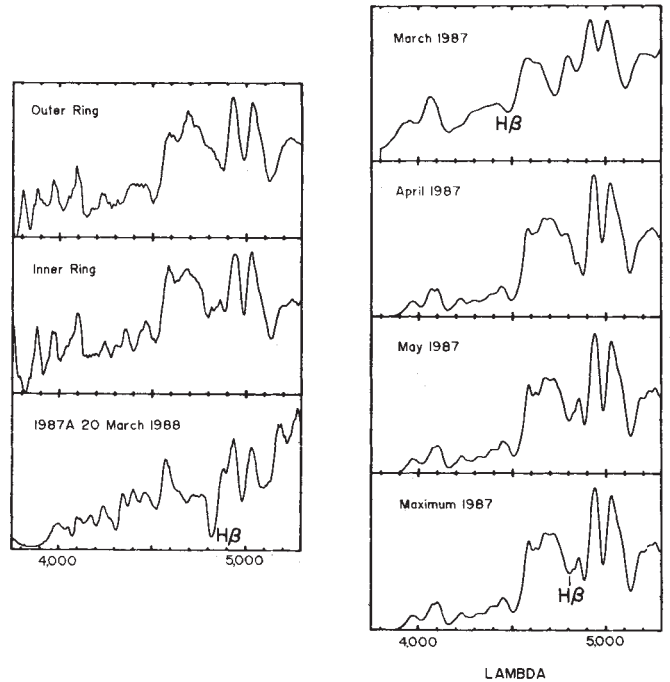


Fig. 3 Spectrophotometry of the echo compared with average spectrophotometry from March, April and May 1987 and from the 71 days of SN1987A's peak luminosity. A long slit of width 1.4 arcsec was positioned tangent to the outer arc, centred at 49.5 arcsec N and 25 arcsec W of the supernova; for the inner arc the slit was centred and 27 arcsec N and 20 arcsec. 100-min exposures were made at each position. The long-slit spectra were reduced to one-dimensional spectra by summing along 10 arcsec of the slit free of stellar contamination; they were sky-subtracted, put on absolute flux and wavelength scales and smoothed by a 20-pixel 'running boxcar'. The left side of the panel shows the inner- and outer-echo spectra and the spectrum of SN1987A on 19 May 1988. The right panel shows the averaged data.

along the line of sight that can be illuminated is $P \sim A\delta t/t$, which is 20% of the distance between the cloud and the supernova. The part of the cloud further away from the observer will reflect light which left SN1987A earlier. The fact that the spectrum of the outer echo is not the average of the whole light pulse must be because the cloud extent is smaller than 20% r .

The colours of SN1987A during the 71 days of peak luminosity were quite constant at $B-V = 1.65$, and $V-R = 0.72 \pm 0.02$. Only after day 101 did they begin to change rapidly. The observed colours of the echo and the spectrophotometry given in Fig. 3 show that the echoes are very blue. This is because the light from the echo is the forward-scattered contribution of the interstellar extinction, which for typical grain properties is very blue²⁷. The constancy of SN1987A's colours during the maximum allows accurate colours and spectrophotometry to give an interesting estimate of the wavelength-dependence of the scattering efficiency. From our spectrophotometry, we find that the ratio of the echo spectra to the best-fitting averaged spectra can be fitted by a power-law function of the form $k\lambda^{-\alpha}$, where $\alpha = 4.9 \pm 0.8$.

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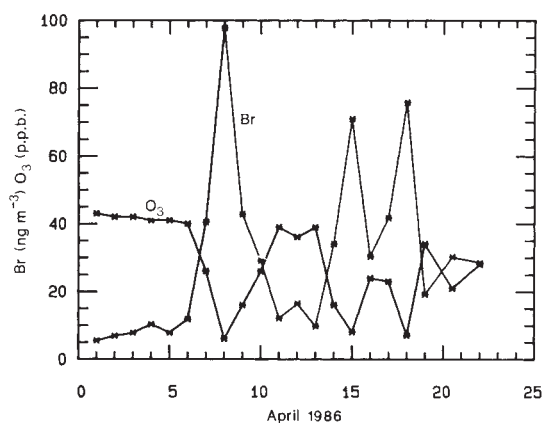


Fig. 1 A comparison of daily mean ground level O_3 and filterable Br(f-Br) concentrations at Alert, Canada, in April 1986 (from Barrie *et al.*²), illustrating the strong inverse correlation between the two parameters.

Ozone destruction and photochemical reactions at polar sunrise in the lower Arctic atmosphere

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There is increasing evidence that at polar sunrise sunlight-induced changes in the composition of the lower Arctic atmosphere (0–2 km) are taking place that are important regarding the tropospheric cycles of ozone, bromine, sulphur oxides¹, nitrogen oxides² and possibly iodine³. Here we focus on recent ground-level observations from the Canadian baseline station at Alert (82.5° N, 62.3° W) and from aircraft that show that ozone destruction is occurring under the Arctic surface radiation inversion during March and April as the Sun rises. The destruction might be linked to catalytic reactions of BrO_x radicals and the photochemistry of bromoform, which appears to have a biological origin in the Arctic Ocean. This may clarify previously unexplained regular springtime occurrences of ozone depletion at ground level in a 10-year data record at Barrow, Alaska⁴, as well as peaks in aerosol bromine observed throughout the Arctic in March and April³. Current information does not allow us to offer more than a speculative explanation for the chemical mechanisms leading to these phenomena.

It has been found in recent years that the Arctic atmosphere is highly polluted during winter because of strong transport from Eurasia to the pole, weak pollutant removal by precipitation and inefficient uptake at the cold, relatively inert Earth's surface⁵. This pollution has been present since at least the turn of the century and has been on the increase from 1950 to 1980 owing to increasing industrial activity^{6,7}. The phenomenon of visibility-reducing air pollution commonly known as 'Arctic

haze' has prompted new measurements of atmospheric composition at high-latitude stations on a long-term basis and periodic intensive airborne research^{8,9}.

Sudden disappearances of O_3 in air at ground level at Alert were first noticed during an intensive study in March 1985 (ref. 10). Volume mixing ratios dropped from 30 to 40 parts per billion (p.p.b.(v.)) to almost 0 p.p.b.(v.) in the time span of a few hours to a day. A more detailed field study during April 1986 confirmed that this was a regular occurrence at this time of year and that a chemical link with bromine existed. The latter was suggested by a strong anti-correlation between daily mean concentrations of O_3 and Br collected on cellulose Whatman 41 filters² (Fig. 1). We have reason to believe that the latter (which will henceforth be referred to as f-Br) is the sum of particulate Br and gaseous HBr. There are indeed indications from measurements at Alert that 50–93% of f-Br is gaseous, probably HBr (ref. 2).

Continuous ground-level O_3 records at Alert between February and June in 1986 and 1987 (Fig. 2) show that, while proceeding from dark winter to sunlit spring, sudden downward departures from the mean concentration curve occurred. The magnitude of the departure paralleled the increase of sunlight at polar sunrise. These data, when combined with observations from a routine weekly aerosol chemistry record at Alert, show that ozone was strongly anti-correlated with f-Br, even when averaged on a weekly basis.

Further evidence that the period of polar sunrise is marked by large variations in lower tropospheric O_3 , and that the observations at Alert are typical of the lower Arctic atmosphere, is offered by 10 years of ground-level O_3 observations at Barrow, Alaska⁴. After polar sunrise in March and April as sunlight increases, not only is there a minimum in the median O_3 concentration observed, but also an absolute maximum in its variability.

Another piece of the puzzle was provided by aircraft observations over the Arctic icecap made during the spring months of March and April^{11–13}. Whenever there was a strong surface radiation inversion, O_3 was depleted below the inversion, whereas fine particle mass was not. In addition, f-Br peaked in the near-surface layer^{14,15}.

The seasonal cycle of f-Br in the Arctic atmosphere has long been a curiosity^{3,16,17}. Excess f-Br over that from windblown dust, sea salt and automobile fuel additives peaks in concentration in the spring months of March and April throughout the North American Arctic. The maximum, which occurs shortly after Arctic sunrise between late March and the end of April, has been tentatively attributed to photochemically induced conversion of gaseous organobromine compounds. The average

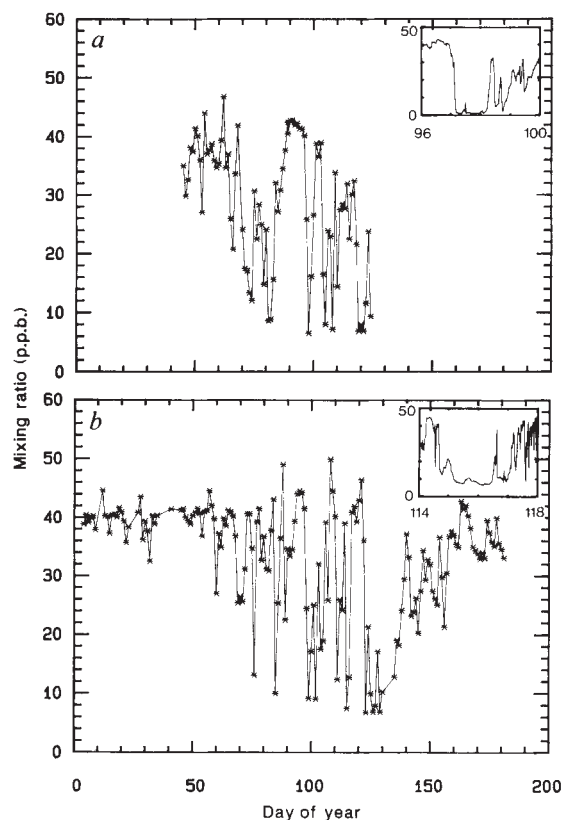


Fig. 2 Daily average concentrations of O_3 observed at ground level at Alert in the Canadian high Arctic during the first half of a, 1986 and b, 1987 (J.W.B.). Note the dip in concentration and the greater variability following polar sunrise, which occurs in early March. The inserted figures show examples of continuous measurements during selected low- O_3 episodes.

concentration of the possible gaseous precursors of f-Br has been estimated for the Arctic and Antarctic lower troposphere by Khalil *et al.* from observations¹⁸. Total alkanobromine concentrations were found to be a factor of 3.6 higher in the Arctic than in Antarctica. Moreover, in contrast to the south polar region, the north has most of its alkanobromine as bromoform (57% versus 15%). This compound ($CHBr_3$) is the most easily photolysed of all the alkanobromine gases. Aircraft observations made in March 1983 suggest that its concentration peaks in the Arctic troposphere below the radiation inversion^{19,20}. More extensive observations during April 1986 confirmed this (Fig. 3), which indicates that bromoform is being produced in the Arctic Ocean, possibly by decay of marine algae such as red benthic algae²¹. The seasonal variation of $CHBr_3$ at Alert during 1986 and 1987 is shown in Fig. 4. The seasonal variation of $CHBr_3$ is somewhat different from the pattern observed at Barrow, Alaska from 1984 to 1987 (ref. 22), but as a result of the limited number of samples per month (3–6 at Alert, 8 at Barrow), it is too early to judge whether these differences are significant. The fact remains that the most rapid decrease in bromoform is observed during polar sunrise. This is suggestive of photochemical activity of this gas.

In what follows, we propose an explanation for the observed depletion of O_3 and associated production of f-Br at polar sunrise in the lower Arctic troposphere. The scenario is as follows. Persistently throughout winter and early spring, the surface boundary layer of the Arctic troposphere over the icecap is isolated from the free tropospheric reservoir of O_3 by a strong radiation inversion. Ozone destruction and f-Br production from gaseous alkanobromines, principally $CHBr_3$, are induced in the boundary layer by sunlight at polar sunrise. The strong inversion

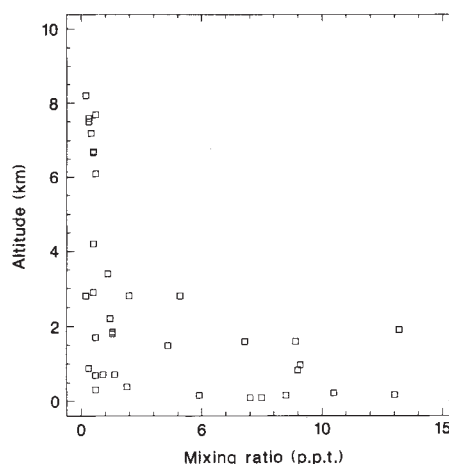


Fig. 3 The vertical distribution of bromoform concentrations in the Arctic atmosphere over the icecap during April 1986 reconstructed from flask measurements from aircraft (R.A.R.).

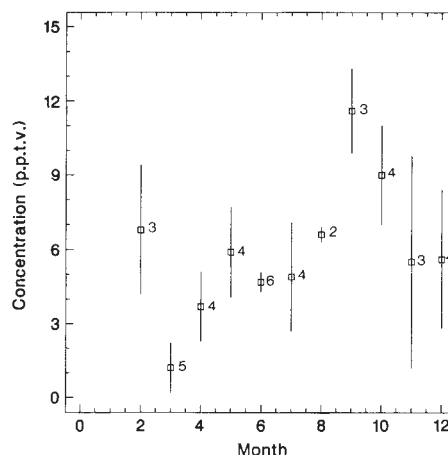


Fig. 4 The seasonal variation of ground level bromoform ($CHBr_3$) mixing ratio at Alert in the Canadian high Arctic obtained from routine weekly flask sample measurements in 1986 and 1987 (R.A.R.). Points on the graph are coded with the number of samples available.

inhibits the replenishment of O_3 from aloft and the dilution of f-Br produced. It also acts to trap organobromines generated by natural sources in the Arctic ocean and thereby enhances the rate of O_3 destruction and f-Br production. At Alert, what we are observing at ground level is the alternating presence of below-inversion air off the icecap and of above-inversion air mixed to the surface in winds coming off the rougher terrain of Ellesmere Island. The former is depleted in O_3 but enriched in f-Br, and in the latter the opposite occurs.

In theory, depletion of O_3 by dry deposition (namely, destruction at the Earth's surface) is possible. But observed dry-deposition velocities of O_3 to snow surfaces²³ are low (0.0006 m s^{-1}). Thus, the time to deplete 90% of ozone from a 1,000 m layer (a typical height for the surface radiation inversion) is about 44 days. This is much longer than the average transit time of an air parcel across the Arctic²⁴. Hence dry deposition appears to be an insufficient sink for surface O_3 . In addition, dry deposition as the main loss mechanism of O_3 does not explain why low O_3 concentrations are not observed during the dark winter.

We favour a chemical explanation (see Table 1), in which ozone destruction occurs by the catalytic cycle of reactions R2, followed by reactions R3a or R3b (Br_2 very rapidly photolyses

Table 1 Chemical reactions postulated to be involved in ozone destruction during polar sunrise involving ozone active bromine and NO_x catalysts

Reaction	Rate coefficient
CHBr ₃ + <i>hν</i>	→ Br + CHBr ₂ $J_1 = 6 \times 10^{-7}$, (R1) → <i>n</i> BrO _x $n = 1, 2 \text{ or } 3$
Br + O ₃	→ BrO + O ₂ $k_2 = 7 \times 10^{-13}$ (R2)
BrO + BrO	→ 2 Br + O ₂ $k_{3a} = 2.5 \times 10^{-12}$ (R3a) → Br ₂ + O ₂ + (<i>hν</i>) $k_{3b} = 7 \times 10^{-13}$ (R3b) → 2 Br + O ₂
BrO + NO	→ Br + NO ₂ $k_4 = 2.5 \times 10^{-11}$ (R4)
BrO + <i>hν</i>	→ Br + O $J_5 = 5 \times 10^{-3}$ (R5)
NO ₂ + <i>hν</i>	→ NO + O $J_6 = 2.5 \times 10^{-3}$ (R6)
O + O ₂ (+M)	→ O ₃ (+M) $k_7 = 2 \times 10^{-14}$ (R7)
NO + O ₃	→ NO ₂ + O ₂ $k_8 = 7 \times 10^{-15}$ (R8)
BrO + NO ₂ (+M)	→ BrONO ₂ (+M) $k_9 = 2 \times 10^{-11}$ (R9)
BrONO ₂ + <i>hν</i>	→ BrO + NO ₂ (?) $J_{10} = 4 \times 10^{-4}$ (R10)
Br + CH ₂ O	→ HBr + CHO $k_{11a} = 7 \times 10^{-13}$ (R11a)
Br + HO ₂	→ HBr + O ₂ $k_{11b} = 8 \times 10^{-13}$ (R11b)
CH ₂ O + <i>hν</i>	→ C + O + H ₂ $J_{12a} = 1.5 \times 10^{-5}$ (R12a) → H + CHO + (2O ₂) $J_{12b} = 6.5 \times 10^{-6}$ (R12b) → 2HO ₂
O ₃ + <i>hν</i>	→ O(¹ D) + O ₂ $J_{13} = 7 \times 10^{-7}$ (R13)
O(¹ D) + H ₂ O	→ 2OH $k_{14} = 2.3 \times 10^{-10}$ (R14)
HBr + OH	→ Br + H ₂ O $k_{15} = 1.0 \times 10^{-11}$ (R15)
BrO + HO ₂	→ BrOH + O ₂ + (<i>hν</i>) $k_{16} = 5.0 \times 10^{-12}$ (R16) → Br + OH

All rate constants, in units of s⁻¹ and cm³ molec⁻¹ s⁻¹, are estimated for the environmental conditions during the measurement period (250 K, one atmosphere pressure) from data in ref. 29. Other reactions involving HO_x, CH₄, CO, and their reactive products are standard. *J*-values are arithmetic averages, in time, diurnally for a three-week period centred on 1 April and, in space, for latitudes 65, 75 and 85° N, calculated with a multiple scattering routine for 400 overhead Dobson units and for average cloudiness conditions.

into two Br atoms). This cycle is moderated by NO_x (NO + NO₂) as a result of reactions R4 and R5, which negate ozone loss by the reformation of ozone through reactions R6 and R7. Similarly, the formation of BrONO₂ in reaction R9 decreases ozone destruction by removing BrO. Reaction R11a of Br with CH₂O also reduces the efficiency of O₃ destruction, but concentrations of CH₂O during early springtime in the Arctic should be low as a result of relatively rapid photolysis (reactions R12a and b). Furthermore, production of CH₂O due to methane oxidation (not shown in Table 1) is inefficient at this time of year because of the low rate of OH production by reactions R13 and R14. We calculate typical CH₂O mixing ratios in the 10–30 parts per trillion (p.p.t.(v.)) range. Preliminary results at Alert in March–April 1988 show that CH₂O and NO_x concentrations are low (<50 p.p.t.(v.)). The Arctic conditions in the lower troposphere may, therefore, correspond to those in the 'ozone hole' of the Antarctic lower stratosphere, which likewise are characterized by low NO_x concentrations^{25,26}.

There are two potential sources of BrO_x: reaction R1 (the photolysis of bromoform) or reaction between OH and HBr (R15). Recent independent measurements of the absorption cross-sections of CHBr₃ by S. Penkett and R. A. Cox (Harwell, UK) and by W. Schneider and G. Moortgat (Mainz, FRG), and detailed radiative transfer calculations for typical conditions on 1 April indicate an average diurnal photolysis rate constant of 6×10^{-7} s⁻¹ north of 65° N. Photolysis leads directly to the formation of one Br atom; subsequent oxidation reactions of the CHBr₂ fragment, probably by CBr₂O photolysis, may produce two additional BrO_x (Br + BrO) radicals.

Selected results of model simulations for the spring period using the reactions outlined in Table 1 are shown in Table 2. The right-hand column is the per cent change in ozone after three weeks. A constant volume mixing ratio of CHBr₃ of about 8 p.p.t.(v.) was used. This is consistent with aircraft observations over the icecap in Fig. 4. Ozone depletion depends on the amount of HBr and, for concentrations greater than a value in the 10–100 p.p.t.(v.) range, on that of NO_x. In the case of the f-Br captured by the filter being representative of gaseous HBr (~30 p.p.t.(v.) HBr), our model predicts 50–60% ozone destruc-

Table 2 Results of model calculations simulating chemical reactions in the lower Arctic atmosphere during the period 21 March to 11 April

NO _x (p.p.t.(v.))	HBr (p.p.t.(v.))	BrO (p.p.t.(v.))	Br (n cm ⁻³)	OH (n cm ⁻³)	O ₃ (p.p.b.(v.))	Per cent change in O ₃
1	30	11	3.4×10^6	7.1×10^4	16	-60
10	30	9.6	3.3×10^6	7.8×10^4	19.2	-52
100	30	6.1	1.9×10^6	1.7×10^5	36.3	-9
1	0	5.6	1.5×10^6	7.5×10^4	30	-25
10	0	4.4	1.2×10^6	9.3×10^4	32	-20
100	0	1.0	3.7×10^5	9.0×10^4	45	+12.5

Only those results for initial volume mixing ratios of 40 p.p.b.(v.) of O₃, 100 p.p.t.(v.) of CH₂O and three weeks of computation are shown. All units are for volume mixing ratios, with the exception of those for Br and OH, which are given in molecules per cm³. CHBr₃ was kept constant at 8 p.p.t.(v.) whereas HBr was either 0 or 30 p.p.t.(v.) and NO_x either 1, 10 or 100 p.p.t.(v.) It is assumed that three BrO_x molecules are produced for each CHBr₃ molecule photolysed. For BrO and OH, maximum values during the integration period are given. Volume mixing ratios for CH₄ and CO were held constant at 1,700 and 150 p.p.b.(v.) respectively. Note the possibility of relatively high Br atom concentrations approaching 10⁷ per cm³.

tion for 1–10 p.p.t.(v.) NO_x over an integration time of three weeks centred on April. In contrast, for the same NO_x range but in the absence of HBr, 20–25% O₃ destruction is predicted.

The main problem with the assumption of high gaseous HBr concentrations is its source in the atmosphere. If it came solely from CHBr₃ photolysis, a production time of about one month would be required, assuming a maximum possible production of three HBr molecules for each CHBr₃ molecule destroyed. Furthermore, conversion of BrO_x to HBr would be rather inefficient for low CH₂O concentrations. Thus, there may well be other sources of HBr. Altogether, it is clear that there are significant uncertainties that make our explanation of ozone destruction in the lower Arctic atmosphere very tentative, and emphasize the need for more diagnostic measurements. In view of the very efficient O₃ depletion that was observed and which we have considerable difficulty in modelling, it may be asked whether heterogeneous reactions on ice crystals could enhance BrO_x production and catalytic ozone destruction, in the way that probably occurs for ClO_x in the Antarctic lower stratosphere during springtime^{27,28}.

The proposed chemical mechanism implicitly excludes Cl-induced destruction of O₃, which makes the ozone loss distinct from the chemistry that has been postulated to create the 'ozone-hole' during the austral spring in the Antarctic lower stratosphere. In contrast to brominated or iodinated hydrocarbons, chloroform and other chlorinated hydrocarbons do not absorb solar radiation in the lower troposphere, and hence no Cl formation is possible by that route. Furthermore, the modelled ratio of ClO_x to HCl is much lower than the ratio of BrO_x to HBr. This is due to a reaction rate of OH with HCl that is 100-fold lower than with HBr (R15), and to the fact that the reaction of CH₄ with Cl atoms is exothermic whereas that with Br atoms is endothermic.

In conclusion, we would like to re-emphasize that more measurements are needed in the Arctic atmosphere during polar sunrise to elucidate the mechanism of ozone destruction. The Arctic atmosphere is a unique laboratory in which to study the chemical behaviour of anthropogenic and natural substances in the presence and absence of light. There is much to be learned from studies on this part of the globe about the biogeochemical cycles of ozone, nitrogen and sulphur oxides, as well as the cycles of the halogens Br and I.

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Crystal structure of the 80 K superconductor YBa₂Cu₄O₈

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Recently a new Y–Ba–Cu–O superconducting oxide has been prepared as a distinct phase in thin films^{1–3}. The new phase was first seen as a defect structure in bulk samples of YBa₂Cu₃O₇ (refs 4–7) and found to contain an additional copper layer⁷. The proposed defect structure has a *c*-axis spacing of ~27 Å and a cation ratio of YBa₂Cu₄Y–Ba–Cu–O films containing a majority phase with a 27 Å unit cell have been proposed as evidence of a distinct phase with the same structure as the defects (ref. 1 and K. Char *et al.*, unpublished results). Films of the new phase superconduct with a transition temperature of 80 K (K. Char *et al.*, unpublished results and refs 2 and 3). We show by X-ray crystallography of a film containing 85% of the new phase that the stoichiometry can be written as YBa₂Cu₄O₈. The new structure differs from YBa₂Cu₃O₇ in that the single, linear Cu–O chain parallel to the *b*-axis has been replaced by a double Cu–O chain with edge-sharing, square-planar oxygen coordination.

Zandbergen *et al.*⁷ analysed the planar defects in YBa₂Cu₃O₇ by high-resolution electron microscopy and reported a defect consisting of a “CuO double-layer intercalation”. A structure with a tetragonal symmetry was suggested where the additional copper layer is shifted by half-a-lattice spacing along the *a*-axis and the *b*-axis. Marshall *et al.*¹ prepared a film of nominal metal composition Y_{1.4}Ba_{2.2}Cu₄, the majority phase of which had a unit cell with a *c*-axis spacing of 27.2 Å, and so was postulated to be a distinct phase with the same structure as the planar defects seen earlier^{4–7}. The films were shown to have an orthorhombic *A*-centred cell and a modification of the defect structure with orthorhombic *Ammm* symmetry was proposed (ref. 1 and K. Char *et al.*, unpublished results). In the orthorhombic model the extra copper cation is displaced along the *b*-axis only, rather than along both the *a*- and *b*-axes. None of the previous studies reports measurements of the oxygen stoichiometry of the new phase.

Here we measure the structure and composition of the new phase, YBa₂Cu₄O₈, by X-ray diffraction from a film. Films were prepared by pulsed excimer laser evaporation of a composite target containing BaF₂–Y₂O₃–CuO, as described earlier³. Films

Table 1 Measured and calculated F^2 for YBa₂Cu₄O₈

<i>h</i>	<i>k</i>	<i>l</i>	F_o^2	F_c^2
Mo radiation				
0	0	4	976	991
0	0	6	294	245
0	0	8	1,011	974
0	0	10	2,790	2,794
0	0	12	8,698	8,814
0	0	16	2,688	2,931
0	0	22	1,194	1,125
0	0	26	2,793	2,010
0	0	30	497	305
0	0	32	111	78
Cu radiation				
0	0	2	926	1,768
0	0	4	3,729	3,629
0	0	6	2,283	1,674
0	0	8	6,296	5,505
0	0	10	9,459	7,974
0	0	12	23,775	24,427
0	0	16	8,284	9,826
0	0	18	624	774
0	0	20	259	228
1	0	2	831	3,570
1	0	3	1,048	1,939
1	0	4	9,795	10,641
1	0	6	14,888	15,403
1	0	8	36,378	35,259
1	0	9	7,717	6,603
1	0	10	883	262
1	0	11	543	372
1	0	12	403	191
1	0	15	2,477	4,010
1	0	16	3,811	3,801
1	0	18	2,993	4,520
1	0	19	3,443	4,356
1	1	3	11,919	14,495
1	1	5	1,215	134
1	1	9	8,584	6,353
1	1	11	12,931	12,088
1	1	13	5,418	5,808
1	1	15	30,868	30,305
0	1	8	41,235	40,955

were grown on a {100} face of SrTiO₃ and found to be oriented with the *c*-axis of the film normal to the substrate. The film used in this study had a thickness of 6,000 ± 500 Å and a second-phase contamination of YBa₂Cu₃O₇ of 15%. Resistance measurements indicated an onset of superconductivity at 82 K, a zero resistance at 77 K, and a width (10–90%) of 3 K (ref. 3). The film *a* and *b* lattice parameters are about equal, with a value of 3.86 Å (as compared with 3.905 Å for SrTiO₃). The *c*-axis of the film is 27.24 Å, a value close to 7*c* of SrTiO₃. The observed selection rules produced diffraction with *l* = 2*n* + 1 absent if *h* and *k* were even, and *l* = 2*n* absent if *h* and *k* were odd, and are not consistent with any tetragonal space group. We interpret the selection rules as evidence of diffraction from individual orthorhombic *A*-centred domains rotated by 90° about the *c*-axis. Such domains with a lateral extent of ~100 μm have been observed before^{1,3}. Although the presence of oriented domains on a substrate do not necessarily imply twinning in the crystallographic sense, we will use conventional analysis of diffraction from a twinned crystal in the discussion that follows.

Because YBa₂Cu₄O₈ is only available as a distinct phase in films, a determination of the structure involves problems not encountered with bulk materials. The primary problem is a small data set containing no Friedel pairs. Additional data were collected using both Mo and Cu Kα radiation from a rotating anode source (repeated observations with Mo radiation provide further structural information because of anomalous dispersion.) The film was mounted on a four-circle diffractometer with Bragg–

Brentano (reflection) geometry. A single bent graphite monochromator maximized the flux by focusing the incident X-rays in the vertical plane. A flat graphite analyser crystal was used on the diffracted beam to provide sufficient resolution to resolve the 27.2 Å *d*-spacing along *c*. A slit after the monochromator restricted the size of the illuminated spot on the film so that the spot would not extend beyond the edges of the sample at low scattering angles. An additional slit on the diffracted beam was used to maintain a symmetric resolution function.

The sample was mounted such that the (001) direction was parallel to the ϕ axis of the diffractometer. This geometry allowed all reflections to be reached with incident and diffracted rays that were symmetric with respect to the film surface ($\omega = 0$). The data were collected by $\theta/2\theta$ scans along (001), (101) and (111). At each point of the scan, counts were acquired with stationary 2θ while moving ω continuously $\pm 2^\circ$ (time-of-flight). Each reflection was bounded by a total of 101 points with an interval in 2θ of 0.03° . The widths of the observed peaks were $\sim 0.6^\circ$. Each scan was fitted to a gaussian plus a background, and the integrated intensity was corrected for Lorentz, polarization, absorption and volumetric effects. An additional (011) reflection was measured to establish the twinning ratio. All data used in the analysis are listed in Table 1. Two of the reflections observed at low scattering angles, the (002) and (102), were used in the refinement, even though the observed intensity was probably too low as a result of the divergence in the area of the illuminated spot as described above.

The space group was assigned as orthorhombic *Ammm*, based on the observation of *A*-centred domains^{1,3} and an assumed centre of symmetry. (Note that the lack of Friedel pairs in the data set prevents the unambiguous assignment of a centrosymmetric space group.) The Patterson function of (001) indicate a metal atom separation of $\Delta z \approx 0.07$. Refinement of positions and occupancies gave a model with the largest cavity occurring at $z \approx 0.135$. The electron density map showed that this position also had the largest number of electrons and so was assigned to Ba, with all other atoms given positions consistent with those in $\text{YBa}_2\text{Cu}_3\text{O}_7$ in the *A*-centred habit. This procedure resulted in the same cation arrangement proposed earlier (ref. 1 and K. Char *et al.*, unpublished results). The positional parameters were refined to $R = 11.9\%$, where

$$R = [\sum (|F_m| - |F_c|)^2 / \sum F_m^2]^{1/2}$$

and F_m , F_c , are the measured and calculated structure factors respectively. The relationship between *A*- and *B*-centred domains was refined with the twinning law $|F_c| = [xF_{hkl}^2 + (1-x)F_{khl}^2]^{1/2}$, where $x = 0.463(5)$.

Oxygen atoms were added to the structure in a manner consistent with the structure of $\text{YBa}_2\text{Cu}_3\text{O}_7$ such that every Cu atom had a sixfold octahedral polyhedron with an overall composition

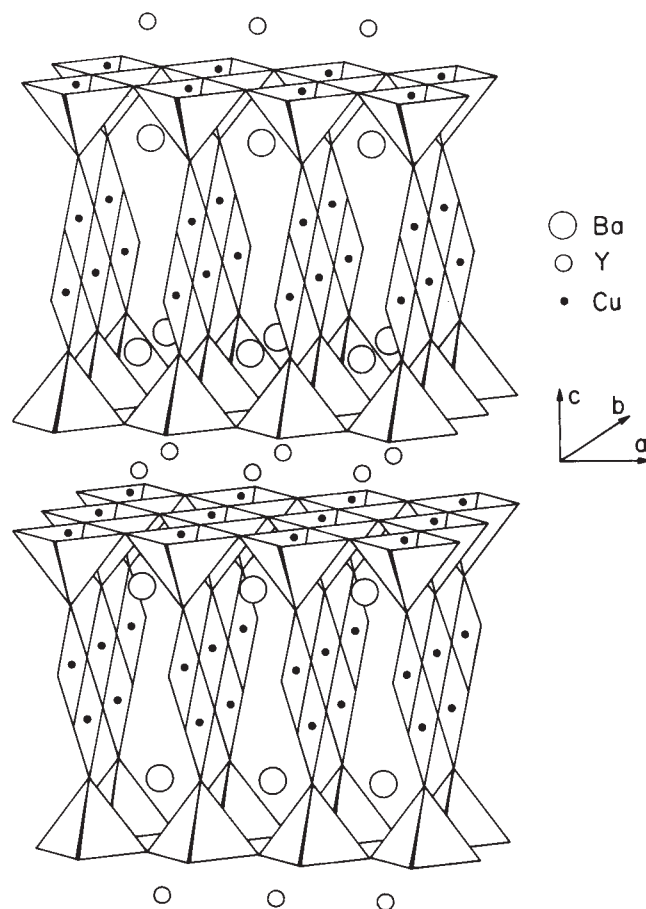


Fig. 1 The structure of $\text{YBa}_2\text{Cu}_4\text{O}_8$. The oxygen coordination polyhedra are drawn using average oxygen positions that eliminate the slight puckering of the Cu-O layers and Cu-O double chains.

of $\text{YBa}_2\text{Cu}_4\text{O}_{11}$. Each oxygen atom was then refined in turn for both position and occupancy. For two of the oxygen sites, a refinement of both the position and the occupancy produced non-convergent refinements. The first site located at 0,0,0 (the Y layer) contains one atom, and the second site located at $\frac{1}{2}, 0, 0.22$ (between the Cu-O double chains) contains two atoms. A refinement of the occupancy of these sites while holding the position fixed resulted in an occupancy within one standard deviation of zero. All other oxygen atoms refined normally. A single Debye-Waller factor was used for all atoms, resulting in $\mu = 0.225(10)$ Å. Refinement of the final model produced $R = 4.0\%$, giving a composition of $\text{YBa}_2\text{Cu}_4\text{O}_8$. The small *R*-factor indicates that the structure is well determined with average positional errors for Ba, Cu and O of 0.0012, 0.0019 and 0.076 Å respectively. The ratio of the observations to the refined parameters (3.74) is somewhat smaller than one would prefer if the sample were a bulk single crystal. As expected, the smaller number of electrons on the oxygen atoms results in the largest positional errors on the oxygen sites. Neutron scattering would be a better probe of the oxygen sub-lattice, however, the lack of bulk samples $\text{YBa}_2\text{Cu}_4\text{O}_8$ prevents such a study.

The crystal structure of $\text{YBa}_2\text{Cu}_4\text{O}_8$ is schematically shown in Fig. 1 and contains an atomic arrangement similar to the planar defect model⁷ and the same as the orthorhombic modification of the defect model suggested by film studies (ref. 1 and K. Char *et al.*, unpublished results). The structure can be compared with the structure of both $\text{YBa}_2\text{Cu}_3\text{O}_7$ (refs 8-13) and $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ (ref. 14). We will first discuss the structure as it compares with $\text{YBa}_2\text{Cu}_3\text{O}_7$. The $\text{YBa}_2\text{Cu}_4\text{O}_8$ unit cell can be considered as two $\text{Ba}_2\text{YCu}_3\text{O}_7$ cells joined chain-to-chain with the second cell displaced $b/2$ along b_0 . This results in the single Cu-O chains of $\text{YBa}_2\text{Cu}_3\text{O}_7$ being replaced by a double chain

Table 2 $\text{YBa}_2\text{Cu}_4\text{O}_8$ atomic positional coordinates

Orthorhombic cell			
$a = 3.86(1)$ Å, $b = 3.86(1)$ Å, $c = 27.24(6)$ Å			
Space group <i>Ammm</i> (No. 65) $Z = 2$			
$\mu = 968 \text{ cm}^{-1}$, $d_{\text{calc}} = 6.11 \text{ g cm}^{-3}$			
39 observed reflections, $R = 0.040$			
Atom*	<i>x</i>	<i>y</i>	<i>z</i> × 10 ⁴
Ba	$\frac{1}{2}$	$\frac{1}{2}$	1,347 (5)
Y	$\frac{1}{2}$	$\frac{1}{2}$	0
Cu1	0	0	2,135 (8)
Cu2	0	0	621 (8)
O1	0	0	1,465 (28)
O2	$\frac{1}{2}$	0	490 (41)
O3	0	$\frac{1}{2}$	570 (30)
O4	0	$\frac{1}{2}$	2,159 (29)
□1	$\frac{1}{2}$	0	2,135
□2	0	0	0

* Vacancy denoted by □.

Table 3 Main interatomic distances in $\text{YBa}_2\text{Cu}_4\text{O}_8$ and $\text{YBa}_2\text{Cu}_3\text{O}_7$

Atoms*	$\text{Ba}_2\text{YCu}_4\text{O}_8$	$\text{Ba}_2\text{YCu}_3\text{O}_7^\dagger$
Cu1-Cu1	2.77 (3)	
Cu2-Cu2	3.38 (4)	3.3698 (13)
Cu1-O1	1.82 (6)	1.858 (4)
Cu1-O1	1.82 (6)	1.858 (4)
Cu1-O4	1.92 (7)	1.942
Cu1-O4	1.931 (2) × 2	1.942 × 2
Cu1-□1	1.930 × 2	1.913 × 2
Cu2-O1	2.30 (6)	2.297 (4)
Cu2-O2	1.961 (7) × 2	1.9306 (6) × 2
Cu2-O3	1.935 (6) × 2	1.9582 (5) × 2
Cu2-□2	1.690-	1.686
Ba-O1	2.748 (7) × 4	2.7427 (5) × 4
Ba-O2	3.02 (7) × 2	2.974 (3) × 2
Ba-O3	2.87 (6) × 2	2.952 (3) × 2
Ba-O4	2.93 (5) × 2	2.8863 (6) × 2
Ba-□1	2.884 × 2	2.905 × 2
Y-O2	2.35 (5) × 4	2.390 (2) × 4
Y-O3	2.48 (5) × 4	2.410 (2) × 4
Y-□2	2.729 × 4	2.726 × 4

* Vacancy denoted by □.

† Data from ref. 13.

of Cu-O to form an edge-sharing, square-planar network with oxygen atoms bonded to copper atoms along both z and y . As each oxygen of the double chain is bonded to three copper atoms rather than to two, $\text{YBa}_2\text{Cu}_4\text{O}_8$ probably does not have the variable oxygen stoichiometry seen in $\text{YBa}_2\text{Cu}_3\text{O}_7$. This square-planar arrangement of copper and oxygen is found in a number of other copper oxides¹⁵, such as SrCuO_2 , and this is probably the reason why the additional copper is incorporated by means of a parallel, double chain rather than by linear chains along both a and b . Apart from the zig-zag arrangement of copper atoms, all other interatomic distances in $\text{YBa}_2\text{Cu}_4\text{O}_8$ are almost identical to those of $\text{YBa}_2\text{Cu}_3\text{O}_7$, as can be seen in Table 3.

The structure of $\text{YBa}_2\text{Cu}_4\text{O}_8$ can also be compared with that of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ ($T_c = 84$ K). The cation framework of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ is similar (although not identical) if Ca is replaced by Y, Bi by Cu, and Sr by Ba. Both compounds have two Cu-O planes separated by a cation plane (Y or Ca) with oxygen vacancies and both have similar superconducting temperatures. Differences in the structures are found in both cation positions and the oxygen occupancy of the Cu or Bi bilayer region. In $\text{Ba}_2\text{YCu}_4\text{O}_8$, oxygen-deficient fourfold planar coordination of copper is present, and in $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ there are sixfold coordinated Bi octahedra with no vacancies. The extent to which the Cu-O double chains act as an electron donor to the Cu-O planes (like the Bi-O octahedra¹⁶ in $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$) or as a metallic chains (as in $\text{YBa}_2\text{Cu}_3\text{O}_7$, see ref. 17) is yet to be determined.

An unusual feature of this structure is the near equivalence of the a and the b lattice parameters, despite the oxygen vacancies between the double chains and the orthorhombic symmetry. The strain provided by the cubic substrate may be important, but it would be unusual for a strain-induced distortion of the intrinsic structure to stabilize the phase. We found no evidence of chemical doping (for example, by Sr, Ti or F) in the film used in this study³, but small amounts of these elements if substituted would not be expected to change the X-ray intensities observed in this experiment.

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Oxidation catalysis on $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$

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Mild oxidation catalysts are used to produce a wide range of industrial chemicals from hydrocarbons, whereas deep oxidation catalysts yield CO_2 and are becoming important in the cleaning of emissions from combustion of carbonaceous fuels. Here we report on a catalyst¹ that facilitates both mild and deep oxidation of toluene in the presence of molecular oxygen and ammonia. At low partial pressures of O_2 a $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$ catalyst is very active in the formation of benzonitrile, but at higher O_2 partial pressures it preferentially catalyses formation of CO_2 . This sharp transition in product selectivity is reversible and occurs at a defined partial pressure of O_2 . At transition the bulk composition of the catalyst is close to $\text{YBa}_2\text{Cu}_3\text{O}_6$ ($x \approx 0$). Increasing the content of lattice oxygen ($x > 0$) makes the catalyst selective for CO_2 formation.

We synthesized $\text{YBa}_2\text{Cu}_3\text{O}_7$ from stoichiometric amounts of Y_2O_3 , BaCO_3 and CuO as described in the legend to Fig. 1. $\text{YBa}_2\text{Cu}_3\text{O}_6$ was similarly prepared and used as a reference. Prepared substances were characterized by X-ray powder diffraction (see Table 1), high resolution electron microscopy and scanning electron microscopy with energy dispersive analysis of X-rays (see Fig. 1). The specific surface area of the starting material used as catalyst was determined with a gravimetric gas adsorption apparatus and found to be $0.1 \text{ m}^2 \text{ g}^{-1}$.

Catalytic activity was investigated in a plug-flow reactor made of Pyrex glass. Reactant flows of O_2 , NH_3 and toluene were mixed with inert N_2 and then passed through a preheater and over the catalyst sample, diluted with acid-washed quartz. Experiments were carried out at 400°C and atmospheric pressure. The composition of the outlet stream from the reactor was analysed on a Varian Vista 6000 gas chromatograph equipped with a flame ionization detector. Freshly prepared $\text{YBa}_2\text{Cu}_3\text{O}_7$ (100 mg) was charged into the reactor and heated under an O_2 -enriched atmosphere to reaction temperature. Preheated reactant mixture was then passed over the catalyst and the partial pressure of O_2 was varied from high to low while the partial pressures of NH_3 and toluene were kept constant. At zero O_2 partial pressure, the experiment was run for 40 min to reduce the catalyst; the partial pressure of O_2 was then varied in the opposite direction up to 8.65 kPa, where it was kept constant for about 2 h. The main reaction products were CO_2 and benzonitrile, although trace amounts of CO and benzaldehyde were also formed.

In Fig. 2a, as the partial pressure of O_2 is varied from high-pressure to the low-pressure region, rates for formation of products showed little or no change down to ~ 8.65 kPa (curves 1 and 2). After reduction at zero O_2 partial pressure, the rate for

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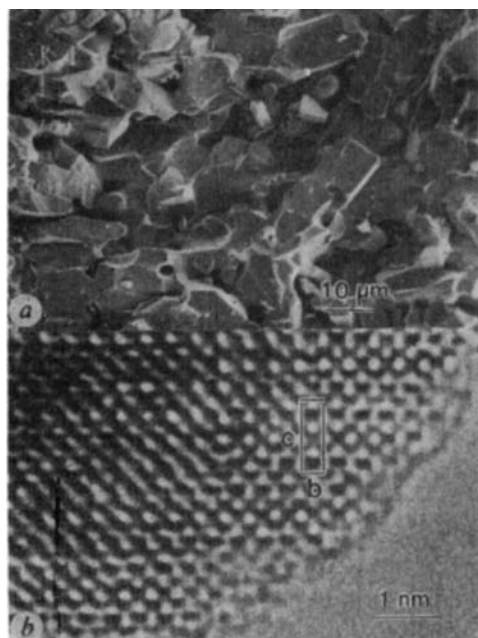


Fig. 1 *a*, Scanning electron micrograph (secondary electron image, JSM-840A) of a fracture surface on a catalyst grain. *b*, Crystal structure image along [100] (or [010]) of a thin grain recorded at Scherzer focus with a 400 kV transmission electron microscope (JEM-4000EX) at a resolution of 1.6 Å. The different cation positions of $\text{YBa}_2\text{Cu}_3\text{O}_7$ can be properly identified by comparing the unit cell (outlined) with computer simulated images.

Methods. $\text{YBa}_2\text{Cu}_3\text{O}_7$ was prepared from stoichiometric amounts of Y_2O_3 , BaCO_3 and CuO . The powders were carefully ground together with acetone in a mortar. The resulting mixture, pressed into tablets, was placed in an alumina boat and heated in air at 950 °C for 17 h. After regrinding, the tablets were treated in a stream of O_2 at 950 °C for a further 17 h. After this treatment, the sample was cooled to 180 °C in an O_2 atmosphere for 8 h. The product showed a distinct diamagnetic response when cooled in liquid N_2 and placed in front of an ordinary magnet. This type of material was gently ground and used as a catalyst. $\text{YBa}_2\text{Cu}_3\text{O}_6$ was used as a reference and was prepared in a similar manner, except that the first sintering was performed in air at 900 °C and the second sintering in flowing argon at 700 °C.

nitrile formation increased with increasing O_2 pressure (curve 3), and was well above the level reached when going from high to low pressures. But as the pressure approached 8.65 kPa, the rate of formation of nitrile suddenly decreased and became strongly time-dependent. Simultaneously, the rate for CO_2 formation increased sharply (Fig. 2*b*). After reaching steady state, the activity for total combustion increased 3 to 4 times over the activity before reductive treatment. The catalyst can function in two regions, depending on the O_2 partial pressure: at low O_2

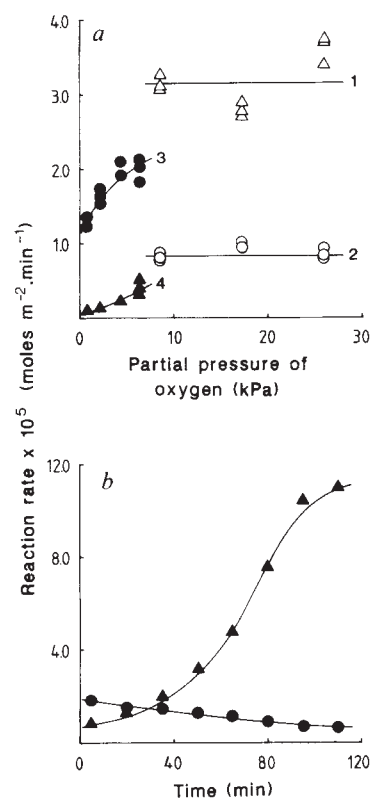


Fig. 2 *a*, Rates for formation of CO_2 (Δ , $\blacktriangle$) and benzonitrile ($\circ$, $\bullet$) as a function of partial pressure of O_2 . Partial pressures of NH_3 and toluene were constant at 2.58 kPa and 0.767 kPa respectively. Open symbols, data obtained when the partial pressure of O_2 was varied from high to low; filled symbols, data obtained after reductive treatment at zero partial pressure of O_2 . *b*, Reaction rates versus time-on-stream at 8.65 kPa partial pressure of O_2 . Partial pressures of NH_3 and toluene as in *a*.

pressures it is active and selective for nitrile formation, but at high O_2 pressures it acts as a total combustion catalyst. After use, the catalysts were quenched from 400 °C to room temperature at a rate of 80 °C min^{-1} and removed from the reactor. This rate of quenching is sufficiently high for the samples to have a representative content of lattice oxygen.

Powder diffractographs were recorded with a Guinier-Hägg camera equipped with a bent quartz crystal monochromator. Unit-cell parameters were determined by least-squares refinement using $\sin^2\theta$ values and a wave length of 1.54056 Å ($\text{CuK}\alpha_1$). Silicon (cubic, $a = 5.43088$ Å) was used as an internal standard. Results are presented in Table 1. The length of the c axis was used to estimate the content of lattice oxygen in the catalysts. This length expands by 0.15 Å (at room temperature) when the oxygen content is reduced from 7 to 6 oxygen atoms per unit cell². As the lattice constants in Table 1 are similar to those reported for the end-components^{3,4}, it can be concluded that the sample prepared in an O_2 atmosphere has a composition close to $\text{YBa}_2\text{Cu}_3\text{O}_7$, whereas the other three samples all have a composition close to $\text{YBa}_2\text{Cu}_3\text{O}_6$. The freshly charged catalyst thus contains 7 oxygen atoms per unit cell, but at zero O_2 pressure the content of oxygen in the lattice drops to 6 oxygen atoms per unit cell. This composition is in principle retained when O_2 is turned on again. In another series of experiments, a CO_2 -selective catalyst, which was not subjected to zero partial pressure of O_2 like the catalysts in Table 1, was found to be tetragonal, with $c = 11.764(1)$ Å. An approximate composition of $\text{YBa}_2\text{Cu}_3\text{O}_{6.4}$ was established by interpolation. A catalyst with $x > 0$ is thus CO_2 -selective. The c repeat of the CO_2 -selective catalyst in Table 1 is also slightly shorter than that of the nitrile-selective catalyst. This again implies a higher oxygen

Table 1 Unit cell parameters of $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$ phases and number of single-indexed powder diffraction lines used in least-squares refinement

Sample:	Fresh catalyst $\text{YBa}_2\text{Cu}_3\text{O}_7$	Nitrile selective catalyst	CO_2 selective catalyst	Reference material $\text{YBa}_2\text{Cu}_3\text{O}_6$
Preparation:	In O_2	From reactor	From reactor	In argon
Symmetry:	Orthorhombic	Tetragonal	Tetragonal	Tetragonal
$a/\text{Å}$	3.8165(4)	3.8560(2)	3.8568(2)	3.8577(2)
$b/\text{Å}$	3.8843(4)			
$c/\text{Å}$	11.680(1)	11.837(1)	11.8255(8)	11.829(1)
Number of lines:	21	23	24	23

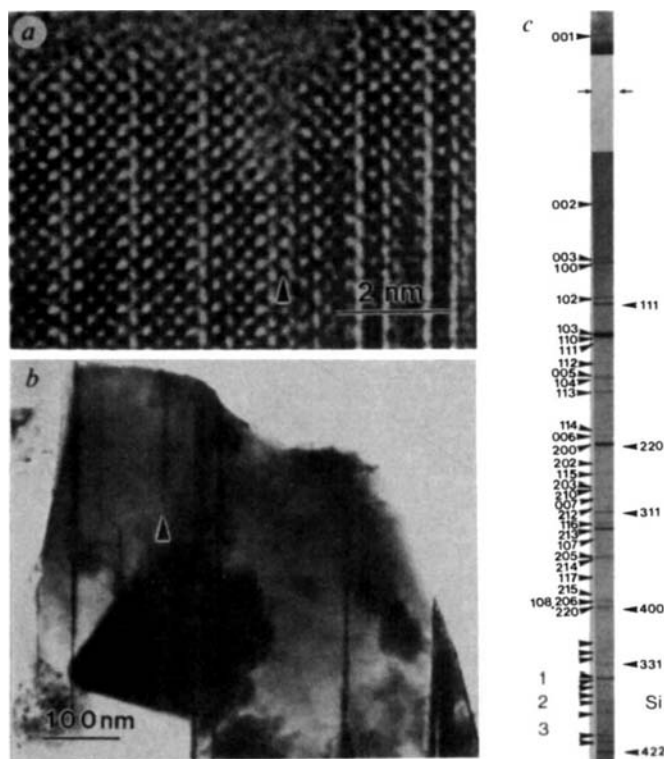


Fig. 3 *a*, Crystal structure image along [100] ([010]) of a $\text{YBa}_2\text{Cu}_3\text{O}_7$ crystal before using it as catalyst, showing a typical, but rare, structure defect (arrowed) on the plane containing chains of corner-sharing CuO_4 squares. *b*, After catalytic ammoxidation such defects have grown wider and become common in most grains of the tetragonal $\text{YBa}_2\text{Cu}_3\text{O}_6$ phase. One terminated defect is marked with an arrow in the image taken with [100] as zone axis. *c*, X-ray powder diffractograph of a catalyst after use for a period of 3 days. Only lines from tetragonal $\text{YBa}_2\text{Cu}_3\text{O}_6$ and the Si standard are present.

content for the CO_2 -selective catalyst. It can be argued that the difference in this case is not large enough to be significant, but the same trend in *c* repeats has now been observed in three additional series of experiments.

The sharp transition at 8.65 kPa is reversible and we found that NH_3 is necessary for it to occur. In the absence of NH_3 , the catalyst is CO_2 -selective at all pressures of O_2 investigated. Comparison of activity and selectivity with other oxide catalysts such as V_2O_5 , used for oxidation and ammoxidation of toluene to benzaldehyde⁵ and benzonitrile⁶ respectively, shows $\text{YBa}_2\text{Cu}_3\text{O}_6$ to be superior. Also, the activity of this catalyst for total combustion is of the same order as CuO and Co_3O_4 (ref. 5) especially in the absence of NH_3 .

The nitrile- and the CO_2 -selective catalysts (Table 1), both used over a period of 10 hours, were shown to consist of a single phase by X-ray powder diffraction, so no decomposition of the catalysts could be detected by this method. Examination of the catalyst before and after use in the reactor by high-resolution transmission electron microscopy, on the other hand, reveals an increase in the number of crystal structure defects on the (001) plane⁷⁻¹⁰, see Fig. 3. It has been reported that water vapour can cause the decomposition of $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$ (refs 7 and 11) by (001) planar defects⁷. Water vapour is one of the products formed in the catalytic oxidation processes.

To assess the longevity of a $\text{YBa}_2\text{Cu}_3\text{O}_7$ catalyst, it was used under the varying conditions described above for a period of 3 days. Once a steady state had been reached, no change with time in the formation of products could be detected. X-ray powder diffractographs of the used catalyst showed lines corresponding exclusively to a tetragonal $\text{YBa}_2\text{Cu}_3\text{O}_6$ phase (Fig.

3c). It is thus unlikely that any significant decomposition of the catalyst will appear during further use.

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Evidence from cathodoluminescence for non-volcanic origin of shocked quartz at the Cretaceous/Tertiary boundary

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Bohor *et al.*¹ have described shocked quartz, displaying one or more sets of planar elements indexed to rational crystallographic planes, associated with anomalous siderophile abundances at the Cretaceous/Tertiary (K/T) stratigraphic boundary in eastern Montana. Shocked quartz has subsequently been shown at several K/T boundary sections around the world, and has been attributed to the effects of impact on crustal rocks²⁻⁷. This impact may have occurred within the scenario of comet (or meteor) collision^{8,9} or, alternatively, shocked quartz at the K/T boundary could be the product of volcanic eruption, rather than bolide impact¹⁰⁻¹⁴. Here we describe a cathodoluminescence study of shocked quartz at the K/T boundary in southeastern Colorado that reveals a diversity of luminescence colours not present in quartz from known volcanic ejecta, but typical of quartz in crustal/supracrustal lithologies. Cathodoluminescence colour diversity indicates that the grains were originally derived from a variety of igneous, metamorphic, and sedimentary rocks subsequently overprinted with planar shock features. These observations lend support to the hypothesis of bolide impact at the end of the Cretaceous period.

Numerous studies have documented an association between general rock type and the cathodoluminescence (CL) colour of its quartz grains. Quartz from extrusive igneous sources luminesces a uniform pale blue¹⁵⁻¹⁸. Quartz from intrusive igneous and high-grade metamorphic rocks generally luminesces darker purple-blue, whereas quartz recrystallized under low-grade metamorphic conditions luminesces reddish-brown¹⁹⁻²⁹. Quartz grains in most sandstones luminesce a mixture of these colours because the grains were derived from a variety of ultimate source rocks. If shocked quartz found at the K/T boundary is volcanic in origin, its cathodoluminescence should be uniform and predominantly pale blue in colour. Alternatively, quartz grains derived from bolide impact on, and ejection of, mixed igneous, metamorphic, and sedimentary rocks should luminesce a variety of colours.

Quartz CL apparently is not affected by shock. Sprunt²⁶ noted no shift in CL colour of quartz experimentally shocked in the

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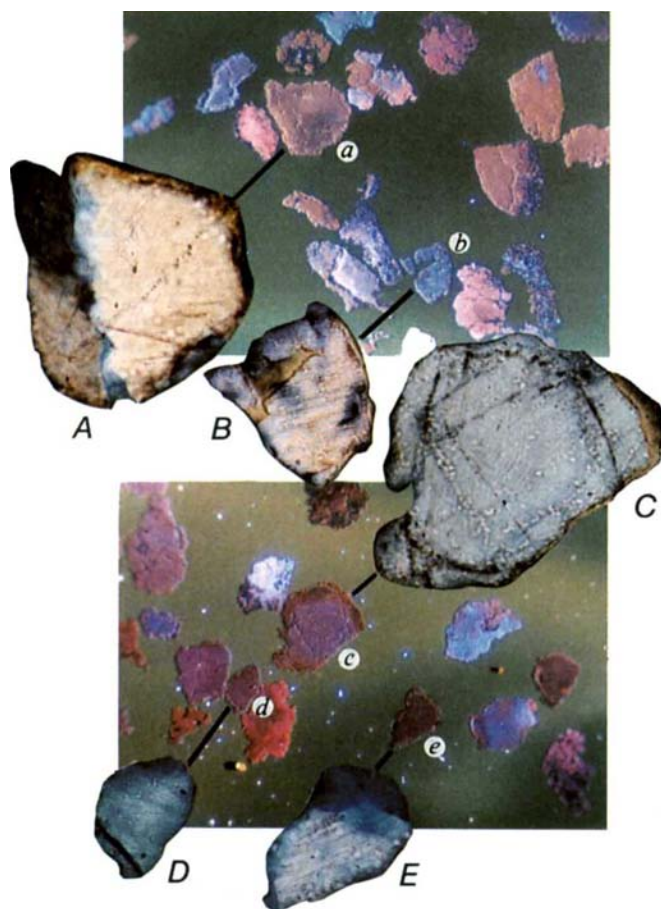


Fig. 1 Mosaic photomicrograph of quartz grain mount. Capital letters represent transmitted light photomicrographs and lower-case letters represent CL images of the same grains (scale bar applies to CL image). *A*, Polycrystalline grain with two sets of shock lamellae in each subcrystal (note differing orientations); *B*, two sets of planar lamellae at low angles to each other; *C*, three sets of shock lamellae; two are readily seen in this photograph; *D*, two sets of lamellae; *E*, one set. In CL, *a*, *d*, and *e* luminesce reddish-brown, whereas *b* and the core of *c* luminesce blue. Note also the quartz overgrowth on *c* (brown CL surrounding medium blue), suggesting that it is a grain from sandstone. The remaining grains in the CL images are not shocked. CL operating conditions were: Nuclide Corp. Luminoscope ELM-2B; 17.5 kV accelerating voltage; 0.1 mA beam current; 3–5 mm diameter beam footprint; Olympus BHSP petrographic microscope with trinocular head; Zeiss UD-20/0.57 objective lens; Kodak VR-1000 colour print film, normal processing.

range 20–22 kbar. Shock-deformed quartz in sandstone from the Vredefort Dome, South Africa and from the Coconino Sandstone, Meteor Crater, Arizona, shows no apparent difference in CL colours when compared to unshocked grains of the same sandstone³⁰.

Shocked quartz at K/T boundary localities in Colorado and New Mexico appears restricted to a 5–8 mm thick horizon just above the iridium-enriched clay layer coinciding with the K/T boundary^{31,32}. Samples from the Clear Creek North site, Raton Basin, Colorado³³ were collected by scraping the exposed surface of the boundary horizon. Samples were washed and treated with HF to remove clay and feldspar. The remaining grains were mounted in epoxy and polished with aluminium oxide granules of 1 µm. Point counting was performed with polarized transmitted light and CL.

Of 1,000 quartz grains counted, 13.7% displayed one or more sets of continuous, planar, closely spaced, parallel shock-deformation lamellae. Of these, approximately 40% were polycrystal-

line grains in which shock lamellae directions were controlled by the differing crystallographic orientations of subcrystals. About 50% of the shocked grains showed more than one set of shock lamellae in flat-stage projection; up to five distinct cross-cutting sets were visible in some grains. The remaining (non-shocked) grains consisted of microcrystalline quartz and chalcedony (46.3%) and polycrystalline + monocrystalline quartz (40.0%).

In CL, 54% of the shocked quartz grains luminesced reddish-brown and the remainder luminesced a variety of blue hues ranging from very dark blue to pale blue (Fig. 1). Fewer than 5% of the blue-luminescing shocked grains (some 3% of all shocked grains) luminesced the pale blue colour that is typical of volcanic quartz. In addition, three shocked grains consisted of a medium-blue-luminescing core surrounded by a dark-brown-luminescing rim. These grains represent originally intrusive igneous (or perhaps high-grade metamorphic) quartz with authigenic quartz overgrowth, and the CL emission appears identical to common quartz sandstone with quartz cement^{34,35}. No correlation was apparent between CL colour and the number of shock lamellae or their orientations.

Shocked quartz at the Raton Basin K/T boundary horizon luminesces a variety of colours and has a very low abundance of the pale blue CL colour typical of volcanic quartz. We conclude that the shocked quartz was not derived from a volcanic source, but from a petrologically diverse source region without substantial volcanic contribution. Most shocked grains were apparently derived from low-grade metamorphic rocks, with a slightly smaller contribution from high-grade metamorphic and intrusive igneous rocks. Rare quartz grains with brown-luminescing rims reflect a minor addition from detrital sedimentary sources. The apparent relative abundances of intrusive (and rare extrusive) igneous, metamorphic, and sedimentary ultimate-source rocks suggested by CL colours of shock-deformed quartz is consistent with a crustal/supracrustal origin for the grains. It does not support a volcanic origin.

It has been proposed^{10–12,14} that an explosive volcanic event might produce shocked minerals in surrounding country rock while perhaps not shocking crystallized minerals suspended within the magma. This could, in theory, lead to a relative enrichment of shocked non-volcanic grains and a paucity of shocked grains in volcanic ejecta. As a partial test of this hypothesis, we examined several thousand quartz grains from the Bishop Tuff (0.7 Myr), the product of one of the largest eruptive events in North America. All of the Bishop Tuff quartz grains luminesced pale blue; no other CL colours were seen. This suggests that crustal contamination is nil, even for an unusually explosive volcanic event. Therefore, the argument of Carter *et al.* and of Hallam^{10–14} seems at variance with conditions in the Bishop Tuff eruption. At present, a more reasonable explanation for the presence of shocked quartz at the K/T boundary is that it represents ejecta resulting from a large bolide impact (or perhaps more than one) on a continent.

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The evolution of flowers with deep corolla tubes

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Some plants have evolved flowers of extraordinary depth, a phenomenon which puzzled Darwin¹. Darwin suggested that the evolution of deep flowers could be a response to a kind of 'race' with pollinating insects: the length of the tongues of pollinating insects could increase as a result of a general size increase, or because it increased their nectar foraging efficiency. As this occurred, plants with relatively shallow flowers could be disadvantaged since pollen transfer, which is effected by physical contact between the pollinator and the anthers or stigma of the plant, could be reduced when the insect tongue is long relative to flower depth. This could lead to the evolution of increasing flower depth which in turn could drive the evolution of a further increase in insect tongue length. Various predictions of Darwin's proposal were tested here for orchid species with deep flowers that are pollinated by moths. It was found that insects do indeed insert their probosces no further than necessary to obtain nectar; that an experimental reduction in flower depth reduces both the male and female components of fitness; and that in natural populations there is a correlation between flower depth and female fitness measured by fruit set. These results all support Darwin's hypothesis to explain the evolution of flower depth.

Floral design ranges from open dish-shaped to stereomorphic with tubes exceeding 30 cm. In 'On the Origin of Species', p. 95, Darwin² reported that certain length relations between the corolla of red clover and the tongue of bees were crucial for successful mutualism and that by the survival of individuals presenting mutual and slightly favourable deviations of structure, a flower and a bee might coevolve. Later, examination of the Madagascar Star Orchid, *Angraecum sesquipedale*, which exhibits nectar-containing spurs 'eleven and a half inches long', made him predict pollination by some giant hawk-moth¹ and (p. 202) that:

"As certain moths of Madagascar became larger through natural selection in relation to their general conditions of life, either in the larval or mature state, or as the proboscis alone was lengthened to obtain honey from the *Angraecum* and other deep tubular flowers, those individual plants of the *Angraecum* which had the longest nectaries (and the nectary varies much in length in some orchids), and which, consequently, compelled the moths to insert their probosces up to the very base, would be best fertilized. Those plants would yield most seed, and the seedlings would generally inherit long nectaries; and so it would be in successive generations of the plant and of the moth."

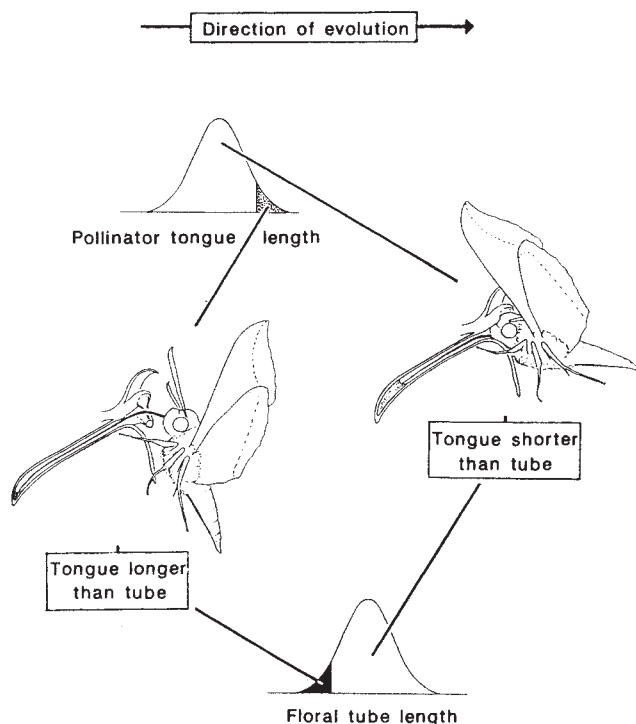


Fig. 1 Modified version of Darwin's hypothesis on the evolution of deep flowers in plants. Long-tongued pollinator individuals are not compelled to contact the floral sexual organs in short-tubed plant individuals, which therefore receive fewer pollinations than the long-tubed individuals that pollinate freely with each other via the pollinators.

A candidate hawk-moth was discovered 40 years later: *Xanthopan morgani praedicta*³. Darwin believed that plant fitness strongly correlated with the length of the nectary exceeding the pollinator tongue length, and this was corroborated in this case. *A. sesquipedale* exhibits an average floral-tube length of 28-32 cm (L.A.N. *et al.*, unpublished results), while tongue length in *X. m. praedicta* reaches ~25 cm (ref. 4). Moreover, tube/tongue ratios > 1 are now known from many deep-flowered plants and their co-evolved pollinator(s)⁴⁻¹⁵. Thus floral tube/pollinator tongue ratios above unity seem universal but Darwin's hypothesis for how flowers become deep has remained untested, although it is potentially of fundamental importance to angiosperm evolution. Its essence is tested here.

Sexual encounters in animal-pollinated plants depend first on the deposition of the pollen grains on the pollinator, and subsequently on the appropriate area of the pollen-vehicle contacting receptive stigmas. At high tube/tongue length ratios, pollen positioning by deep-tubed orchids on their principal insect pollinators is concentrated in a narrow zone at the proboscis base^{4,8,9}. Most pollen is deposited mechanically at a standard position on the bodies of the pollinators as they systematically thrust their probosces maximally into the flowers. Data from orchid populations that occasionally experience chance visits by stray individuals of relatively long-tongued hawk-moth species, indicate that at tube/tongue ratios < 1 pollen positioning is determined by the floral tube length (or, more exactly, the distance between tube extremity to pollen attaching floral structure) (Table 1). Visitors thus do not drive their probosces deeper into flowers than is necessary to imbibe food, so Darwin's prediction about insect behaviour was right.

This information prompts a more explicit formulation of the basic relationship (Fig. 1): the individual pollinators that have the longest tongues will reach the floral tube extremity in those plant individuals that have the shortest tubes and will fail to

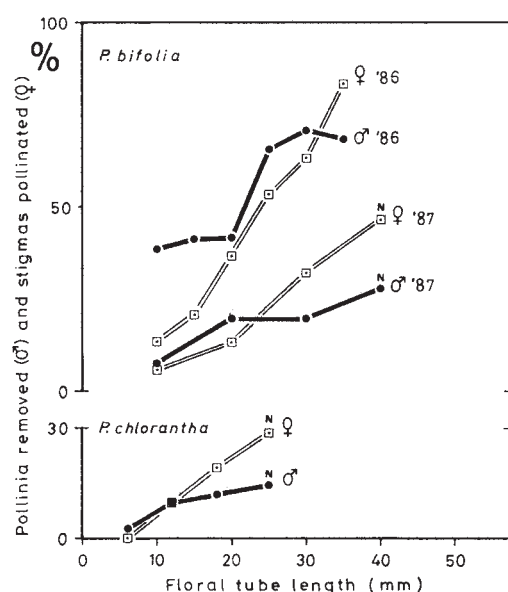


Fig. 2 Experimental shortening of floral tube has a strong negative effect on male (pollinia removal, black bars) and female (stigma pollen receipt, open bars) components of fitness in *P. bifolia* (top) and *P. chlorantha* (bottom). Experiments were performed with series of tube lengths, for *P. bifolia* in 1986; 10, 15, 20, 25, 30 and 35 mm ($n = 30$ per length) (180 flowers) and in 1987; 10, 20, 30 and 40 (natural length) ($n = 59$ per length) (236 flowers) and for *P. chlorantha* in 1987; 6, 12, 18 and 25 (natural length) mm, ($n = 42$ per length) (168 flowers). Three fresh, unvisited flowers per inflorescence were manipulated using tape (1986) or cotton thread (1987). The within-spike positions were alternated. In 1986, the often low nectar-content in shortened spurs was not compensated, but in 1987 the nectar was squeezed up, above the constriction. Patterns in reduction of fitness, however, were similar. Flowers were exposed 10 (*P. bifolia* 1987), 8 (*P. bifolia* 1987) and 6 (*P. chlorantha* 1987) nights to natural pollinators (moths). χ^2 homogeneity tests on data for each sex function and experiment indicate significant effects ($P < 0.025$) of treatment in all cases.

effect contact between their load of 'standardly' positioned pollen-masses and the stigma. Short-tubed individuals therefore are predicted to receive fewer pollinations and to suffer a relatively low fitness through their female function and, if the degree of mismatch reduces access to suitable pollen-mediating vector surfaces, also through their male function. In contrast, long-tubed individuals interpollinate freely and will function as pollen recipients from, though not donors to, short-tubed individuals.

The effect of floral depth was tested in natural populations of *Platanthera bifolia* and *P. chlorantha*. Both orchids have long nectariferous floral spurs and are moth-pollinated⁸. Their pollinia attach to the probosces and eyes, respectively, of nocturnal moths¹⁶. Spurs were experimentally constricted at fixed distances from the entrance. Pollinia removal and pollen receipt (male and female fitness component, respectively) after exposure to natural pollinators showed that spur shortening gives a consistent reduction in fitness for both sex functions (Fig. 2). Similar effects have been recorded in *P. mandarinorum*¹⁷.

In 9 out of the 11 successive reductions in tube length (Fig. 2) pollen export was reduced less than pollen import, indicating that shorter tubed individuals increase their maleness. Pollinia situated further from the base on inappropriately long probosces support this (Table 1).

Fruiting in *P. bifolia* requires prior pollination⁸ and fruit production strongly correlated positively with floral-tube length ($r_{xy} = 0.96$, $F_s = 71.4$, $P < 0.001$) (Fig. 3). The relationship was seemingly curvilinear: below a tube length of ~37 mm fruit production dropped dramatically. With decreasing tube length the proportion of fully swollen fruits fell from 86% to 40% ($n = 134$, 1986). Tongue length, and distance between the pollinia-bearing area and the tip of the proboscis in sampled flower-visitors, showed that within the 37–32 mm range from the tip, pollinia placement abruptly dropped from 91% to 16% (Fig. 3). This coincided exactly with the interval of drastic decrease in mean individual fruit set. Evidently the mechanical accessibility of male mates via the principal local vector *Sphinx ligustri* was the cause. Tube length in *P. bifolia* is variable and longer in the study area than elsewhere⁸, which supports the occurrence of directional selection.

Spur-length differences in *P. bifolia* are under genetic control¹⁸. Thus by mechanically causing assortative plant pollination, long-tongued visitors exert a directional selection pressure. Because the distance between tube tip and anther/stigma, not tube length *per se*, is the critical mechanical variable for vector-mediated sexual encounters, homologous selection in non-orchidaceous plants is predicted to lengthen stamens and styles, as displayed by many flowers^{7,19}. Pollinators' work controls the

Table 1 Pollen placement on visitor hawk-moths

Plant population*, mean and range of floral tube length (mm)	Occasional visitor hawk-moth	Tongue length (mm)	Pollinia placement from tongue tip (mm)
A 22.1, 16–33 ($n = 208$)	<i>Sphinx ligustri</i> ($n = 2$)	35–36.5	21–28 ($n = 7$)
	<i>Hyloicus pinastri</i> ($n = 1$)	25.5	23 ($n = 3$)
B 18.5, 15–22 ($n = 200$)	<i>S. ligustri</i> ($n = 1$)	39	18.5–23 ($n = 20$)
	<i>H. pinastri</i> ($n = 1$)	25.5	17.5–18.5 ($n = 6$)
C 16.1, 13.5–20 ($n = 100$)	<i>Hyles gallii</i> ($n = 2$)	23.5–26	13–18 ($n = 27$)
	<i>Deilephila elpenor</i> ($n = 1$)	22	16 ($n = 3$)
D 19.4, 15–24 ($n = 50$)	<i>S. ligustri</i> ($n = 1$)	36	19.5–22.5 ($n = 22$)
	<i>H. pinastri</i> ($n = 4$)	25.5–33.5	12–23.5 ($n = 12$)

At low floral tube/tongue length ratios this shows that insects do not insert their probosces deeper than necessary into flowers to extract nectar and that, consequently, tube length controls the operative position of pollen on vector. Sample sizes are in brackets and measurements in mm. Data obtained 1972–1980 in Southern Sweden.

* A, *Platanthera bifolia*: Öland, The Alvar; principal pollinator is *Deilephila porcellus*. B, *Gymnadenia conopsea*: Same locality and principal pollinator. C, *G. c. var. densiflora*: Öland, central forest area; principal pollinators are butterflies. D, *G. conopsea*: Gotland, Bäl; principal pollinators are moths with <20 mm tongue length.

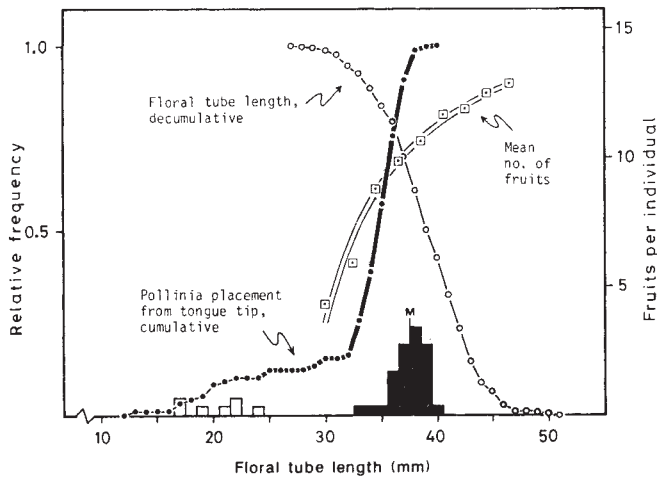


Fig. 3 Floral tube length and pollinia placement pattern on pollen vectors affect differential production of fruit in a population of *P. bifolia* (central forest area on Öland, South Sweden). Heavy black line denotes the contribution from pollinia placement on *Sphinx ligustri*. Effective tongue length (total length minus portion obstructed by palpi) of visiting moths is shown by bars, those representing *S. ligustri* being filled black (*M* = mean length in this hawk-moth). Data obtained 1972–1986. Sample sizes: 349 plants, and 42 moths with a total of 245 pollinia.

pollination pattern in their plants, but long-tongued animals are free to forage both from long and short corolla tubes (as in Table 1). Therefore pollen vectors mainly perform plant breeding, the result of which expresses phenotypic variability that opens up an extended niche for further animal evolution through competition. Food accessibility in longer-than-average tubed individuals tends to increase fitness in longer-than-average tongued, nectar-dependent, individual animals. Such pollinators favour the evolution of longer floral tubes which in turn favours the evolution of still longer tongues in a reciprocal and escalating process. If long-tongued vectors become scarce, the food accessibility pattern may induce shorter-tongued vectors to reverse selection toward shorter tubes.

Darwin's ingenious conclusion from the Madagascar Star Orchid and its predicted phantom sphinx has received attention merely as an anecdote up till now. Data presented here show that he in fact provided the basic idea to a general principle about interactional evolution between animals and flowering plants.

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Projection neurons within a vocal motor pathway are born during song learning in zebra finches

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Many birds learn song during a restricted 'sensitive' period¹. Juveniles memorize a song model, and then learn the pattern of muscle contractions necessary to reproduce the song. Of the neural changes accompanying avian song learning, perhaps the most remarkable is the production of new neurons which are inserted into the hyperstriatum ventralis pars caudalis (HVC)², a region critical for song production³. We report here that in young male zebra finches many of the new neurons incorporated into the HVC innervate the robust nucleus of the archistriatum (RA) which projects to motor neurons controlling the vocal musculature³. Furthermore, far fewer of these new neurons are incorporated into the HVC of either adult males that are beyond the sensitive learning period, or young females (who do not develop song). Thus, a major portion of the vocal motor pathway is actually created during song learning. This may enable early sensory experience and vocal practice to not only modify existing neuronal circuits, but also shape the insertion and initial synaptic contacts of neurons controlling adult song.

Male zebra finches memorize song between 20–65 days after hatching^{4,5}. At about 30 days of age, they begin practicing their own vocalizations, gradually perfecting their song pattern over the next 6–8 weeks. During these periods of song learning in males, new neurons develop and are added to the HVC^{2,6}. In young females, who do not develop song, the number of neurons in the HVC does not increase⁷, and consequently this region is much smaller in adult females than in adult males⁸. In addition to being essential for song production, the HVC contains auditory-sensitive neurons⁹, and may also function in song learning. We were interested in determining which neuronal types are added to the HVC during learning, to understand better how these neurons may contribute to song development.

To identify neurons that develop during song learning, we injected juvenile males with [³H]thymidine daily from 20–40 days of age. Projection neurons within the HVC were then retrogradely labelled by the fluorescent tracer fluorogold (Flg) which was injected directly into either the RA or Area X, the two efferent targets of HVC neurons. Birds were killed at 64 days of age and brain sections were processed for autoradiography. Using ultraviolet (UV) and bright-field illumination, we determined the overall density of Flg-labelled, thymidine-labelled, and double-labelled neurons within the HVC.

In young males, 19.9 ± 1.8% of HVC neurons were labelled by our [³H]thymidine regime (Table 1). This represents the addition of over 18,000 new neurons between 20 and 64 days of age. Remarkably, over half of these neurons project to the RA. Fluorogold injections into the RA retrogradely-labelled 48.2 ± 5.1% of HVC neurons, and of these projection neurons 22.6 ± 1.9% were also labelled with [³H]thymidine, indicating that they were born after day 20 (Fig. 1a). In contrast, less than 5% of the HVC neurons that are born during adolescence project to Area X. Although a substantial number (15.7 ± 2.2%) of HVC neurons were retrogradely labelled following injections of Flg into Area X, only 6.5 ± 1.8% were also labelled with [³H]thymidine (Fig. 1b).

Female zebra finches do not sing and never fully develop the projection from the HVC to the RA¹⁰. We reported previously that fewer new neurons are incorporated into the HVC in young females than in males². The present results suggest that this sex difference in neuronal addition not only leads to the sexual

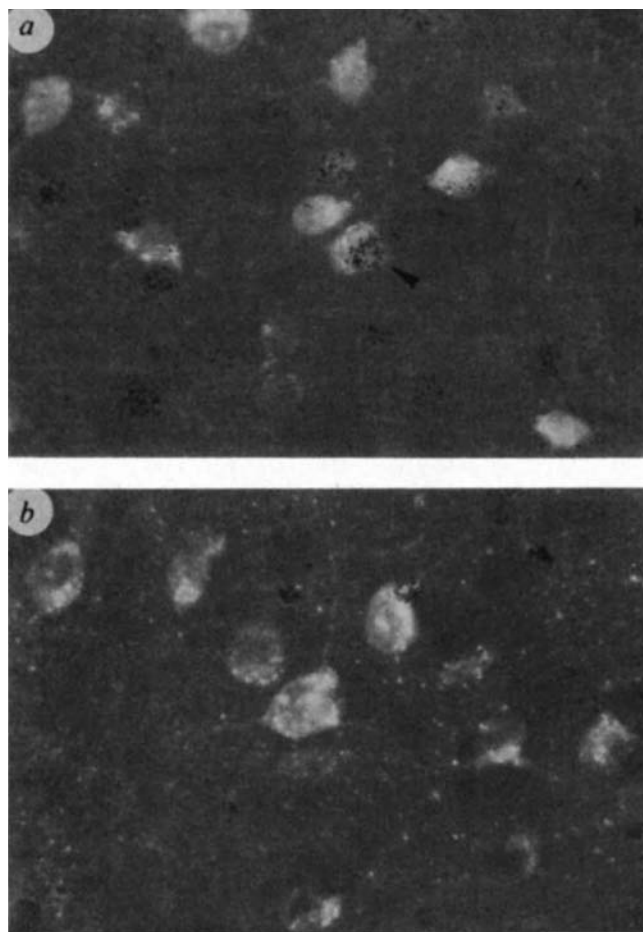


Fig. 1 Photomicrographs of retrogradely-labelled HVC neurons in a 64-day-old male zebra finch following an injection of fluorogold into the RA (a) and Area X (b). Many of the neurons projecting to the RA are labelled by [^3H]thymidine injected between 20 and 40 days of age (arrow in a).

differentiation of HVC neuron number, but also to the development of a sexually dimorphic neural pathway. To investigate this possibility we treated young females with [^3H]thymidine as described above and injected Flg into the RA before killing the birds at day 64. As shown in Table 1, the incidence of thymidine-labelled neurons in the HVC was significantly less in young females than in males ($df=5$, $t=3.56$, $P<0.01$). Likewise, the percentage of Flg-labelled HVC neurons was significantly less in females than in males ($df=5$, $t=4.51$, $P<0.005$), indicating that relatively few of the neurons in the female HVC project to the vicinity of the RA. Most importantly, the incidence of [^3H]thymidine labelling among neurons projecting to the RA was significantly less in young females than in males ($df=5$, $t=5.72$, $P<0.005$). These results argue that the incorporation of new projection neurons into the male HVC during adolescence contributes to the abrupt innervation of the RA that occurs at this time¹⁰. Females may fail to develop this pathway because of their reduced incorporation of new HVC neurons. Although we do not know whether sex differences in the incorporation of neurons into the HVC reflect differences in the rate of neurogenesis, or the death of newly-generated neurons, the rate of HVC cell death is higher in females than in males during this period of development¹¹.

There are now several reports of new neurons being incorporated into the juvenile and adult song system^{2,12,13}, but the relation between neurogenesis and song learning is not clear. Our demonstration that neurons born during song learning in zebra finches contribute directly to the motor pathway controlling

Table 1 Sex and age-related differences in the incorporation of new projection neurons into the HVC

		% Thymidine	% Fluorogold	HVC→RA % Thymidine
Male	65 day ($n=4$)	19.9 ± 1.8	48.2 ± 5.1	22.6 ± 1.9
	Adult ($n=3$)	6.5 ± 1.0	53.9 ± 1.9	1.9 ± 0.1
Female	65 day ($n=3$)	11.9 ± 1.0	20.3 ± 1.6	8.9 ± 0.9

Data shown (means $\pm$ s.e.m.) are the overall incidence of [^3H]thymidine-labelled HVC neurons, the proportion of HVC neurons retrogradely-labelled following an injection of Flg into the RA, and the incidence of [^3H]thymidine labelling among HVC neurons projecting to the RA in juvenile male, juvenile female and adult male zebra finches. Juveniles were injected with $2.5 \mu\text{Ci g}^{-1}$ [^3H]thymidine every 24 h from 20–40 days after hatching. Adult males 120–180 days of age received identical injections. Seventeen days after the last [^3H]thymidine injection, the birds were anaesthetized and the RA was injected bilaterally with 0.02–0.04 μl fluorogold. Seven days later the birds were again anaesthetized and perfused with saline-formalin. Their brains were removed and embedded in paraffin. Coronal sections (10 μm) were dipped in NTB-3 nuclear track emulsion, exposed for 2–4 weeks, developed (Kodak D19) and fixed. Sections through the HVC were first examined under UV illumination to determine the density of Flg-labelled neurons. The number of silver grains over each Flg-labelled neuron was then determined by combining UV and bright-field illumination. Sections were then lightly stained with thionin and the overall neuronal density and incidence of thymidine-labelled neurons were determined. Neurons were considered labelled by thymidine, and thus born after day 20, if the density of silver grains over the cell body exceeded $5 \times$ background grain density.

adult song strengthens the possibility that neurogenesis is an important element of the neural plasticity required for vocal learning. Adult male zebra finches also incorporate new neurons into HVC¹⁴, however, even though they do not change their vocal repertoire after song crystallization. For this reason it was important to determine whether the types of HVC neurons added during song learning are different from those added in adult birds. We injected adult male zebra finches with [^3H]thymidine for 20 days and killed them 25 days later; 6–8 days after receiving an injection of Flg into the RA. As shown in Table 1, the incidence of thymidine-labelled neurons incorporated into the HVC over this time period was significantly less in adult males than in juvenile males ($df=5$, $t=5.99$, $P<0.001$). Moreover, although the percentage of neurons projecting to the RA did not differ between adult and juvenile males, the proportion of these neurons also labelled by [^3H]thymidine was significantly less in adults than in juveniles ($df=5$, $t=9.08$, $P<0.001$). Thus, while most HVC neurons added during song learning innervate the RA, those added in adulthood rarely contribute to this vocal motor pathway. Perhaps HVC neurons produced in adult finches are interneurons, as has been demonstrated for those neurons added to the HVC in adult canaries¹⁵. The behavioural significance of this adult neurogenesis is unclear, but because new neurons are also added to the HVC in adult female canaries (who do not normally sing) their contribution to vocal plasticity is questionable. Instead, Nottebohm has suggested that adult neurogenesis may be important for the restructuring of perceptual memories¹⁶, a task common to both males and females.

During development, auditory experience and vocal practice interact to determine the structure of adult song¹⁷. Thus, avian vocal learning is one of many examples in which the course of neural and behavioural development depends on sensory experiences during an early critical period¹⁸. Because the motor pathway controlling song is actively forming during the sensitive learning period, we may need to expand our view of how early experience shapes this neural pathway. Whereas the prevailing view is that neuronal activity normally influences development by regulating regressive events, such as cell death and synapse elimination^{19–21}, temporal overlap between song development

and the production of new, song-related neurons would allow learning also to tailor the initial organization of vocal pathways. Early exposure to a song model, and subsequent vocal practice, may shape adult song in part by regulating the production and insertion of new neurons, or by influencing the specificity of their initial synaptic connections. An understanding of how such progressive developmental events are influenced by neuronal activity is critical as the insertion of new neurons into functioning neural circuits becomes increasingly accepted both as an aspect of normal ontogeny, and as an experimental approach.

We would like to thank Marc Dorfman, Beth Pinckney and Farida Sohrabji for their technical assistance on this project. The work was supported by Alfred P. Sloan Fellowships to E.J.N. and K.W.N., as well as by the US PHS.

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Pattern formation in the developing eye of *Drosophila melanogaster* is regulated by the homoeo-box gene, *rough*

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Homoeo-box genes play a central role in the regulation of embryogenesis in *Drosophila melanogaster*¹⁻⁶. Their widespread phylogenetic distribution⁷⁻⁹, and the tissue and stage specificity of their expression in other organisms¹⁰⁻¹⁶, argue that they play a general and significant role in animal development. In *D. melanogaster*, all homoeo-box genes characterized to date are involved in major aspects of embryogenesis. We report here the molecular characterization of a *Drosophila* homoeo-box gene that has no apparent involvement in early embryogenesis. The gene appears to be *rough*, a gene implicated in pattern formation in the developing eye^{17,18}. It is expressed in cells within, and posterior to, the morphogenetic furrow, the site of the primary pattern forming events in the developing retina¹⁹⁻²², and also in a region of the brain of the third instar larva. We have found no genetic or molecular evidence of a role for this gene in other aspects of fly development.



Fig. 1 Hybridization *in situ* of a genomic DNA fragment containing homoeo-box homology to polytene chromosomes of the salivary gland of *D. melanogaster*. The cloned fragment was identified by virtue of its homology, under low stringency hybridization conditions (50% formamide, 5 × SSC at 30 °C), to a ³²P-labelled synthetic 33 base oligonucleotide complementary to a consensus homoeo-box sequence. The isolated fragment hybridized to region 97D3-5 of the right arm of the third chromosome, the cytological location of the *rough* gene.

Methods. Hybridization to polytene chromosomes was carried out using biotinylated DNA probes³⁴ and a biotin-alkaline phosphatase detection system (Bresatec, South Australia).

a

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TAA TAA GCT GAT TCC AAC TAG ATA TTA TAT ACG TAT TAA TTG
TAA GGA TAT CAA TGT ATT TGA CTT TCA GTG GCT CGC AGG CGA
*** Leu Ser Val Ala Arg Arg
CGC AAG GAG GGA AGA CAA CGC CGC CAG AGG ACC ACT TTC AGC
Arg Lys Glu Gly Arg Gln Arg Arg Gln Arg Thr Thr Phe Ser
ACA GAG CAG ACG CTT CGC CTG GAG GTG GAG TTC CAT CGG AAC
Thr Glu Gln Thr Leu Arg Leu Glu Val Glu Phe His Arg Asn
GAG TAC ATC TCC AGG AGT CGT CGC TTC GAG CTG GCC GAA ACG
Glu Tyr Ile Ser Arg Ser Arg Arg Phe Glu Leu Ala Glu Thr
CTG CGT CTG ACC GAA ACG CAA ATC AAG ATC TGG TTC CAG AAT
Leu Arg Leu Thr Glu Thr Gln Ile Lys Ile Trp Phe Gln Asn
CGC AGG GCC AAG GAC AAA CGC ATT GAA AAG GCT CAG ATC GAT
Arg Arg Ala Lys Asp Lys Arg Ile Glu Lys Ala Gln Ile Asp
CAG CAC TAC AGG TGG GTT TCC CCT TTA GCT AAT TTG AAT CGT
Gln His Tyr Arg Trp Val Ser Pro Leu Ala Asn Leu Asn Arg
TTT TTC TGT TTC ATA TTT ACA AAG ATA TCT TCG ATT TTG ATT
Phe Phe Cys Phe Ile Phe Thr Lys Ile Ser Ser Ile Leu Ile
GAG TTA AAT AAC ATT AAA TGA ACA TAA ATT AAA GGT TGA TGA
Glu Leu Asn Asn Ile Lys ***
ATA GCT CGT GCA GAG GAT TTT AAC TGG TTA TTT ATA CCT GCA

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b

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ro	Gln	Arg	Arg	Gln	Arg	Thr	Thr	Phe	Ser	Thr	Glu	Gln	Thr	Leu	Arg
Antp	Arg	Lys	Arg	Gly	Arg	Gln	Thr	Thr	Thr	Arg	Tyr	Gln	Thr	Leu	Glu
en	Glu	Lys	Arg	Pro	Arg	Thr	Ala	Phe	Ser	Ser	Glu	Gln	Leu	Ala	Arg
prd	Gln	Arg	Arg	Cys	Arg	Thr	Thr	Phe	Ser	Ala	Ser	Gln	Leu	Asp	Glu

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
ro	Leu	Glu	Val	Glu	Phe	His	Arg	Asn	Glu	Tyr	Ile	Ser	Arg	Ser	Arg
Antp	Leu	Glu	Lys	Glu	Phe	His	Arg	Asn	Arg	Tyr	Leu	Thr	Arg	Arg	Arg
en	Leu	Lys	Arg	Glu	Phe	Asn	Glu	Asn	Arg	Tyr	Leu	Thr	Glu	Arg	Arg
prd	Leu	Glu	Arg	Ala	Phe	Glu	Arg	Thr	Gln	Tyr	Pro	Asp	Ile	Tyr	Thr

	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
ro	Arg	Phe	Glu	Leu	Ala	Glu	Thr	Leu	Arg	Leu	Thr	Glu	Thr	Gln	Ile
Antp	Arg	Ile	Glu	Ile	Ala	His	Ala	Leu	Cys	Leu	Thr	Glu	Arg	Gln	Ile
en	Arg	Gln	Gln	Leu	Ser	Ser	Glu	Leu	Gly	Leu	Asn	Glu	Ala	Gln	Ile
prd	Arg	Glu	Leu	Ala	Gln	Arg	Thr	Asn	Leu	Thr	Glu	Ala	Arg	Gln	Ile

	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
ro	Lys	Ile	Trp	Phe	Gln	Asn	Arg	Arg	Ala	Lys	Asp	Lys	Arg	Ile	Glu
Antp	Lys	Ile	Trp	Phe	Gln	Asn	Arg	Arg	Met	Lys	Trp	Lys	Lys	Glu	Asn
en	Lys	Ile	Trp	Phe	Gln	Asn	Lys	Arg	Ala	Lys	Ile	Lys	Lys	Ser	Thr
prd	Gln	Val	Trp	Phe	Ser	Asn	Arg	Arg	Ala	Arg	Leu	Arg	Lys	Gln	His

Fig. 2 *a*, Nucleotide sequence encoding the open reading frame homologous to the homoeo box. The region of homoeo-box homology is underlined. We have not determined the exon-intron boundaries flanking the homoeo-box sequence, but a consensus splice acceptor site 5' to it is indicated with an arrow. *b*, Comparison of the presumptive amino-acid sequence of the homoeo box in *rough* with those of *Antennapedia*¹, *engrailed*³ and *paired*⁵ showing the extensive homology. Identical amino acids are boxed. Sequence analysis was carried out using the dideoxy chain-termination technique³⁵.

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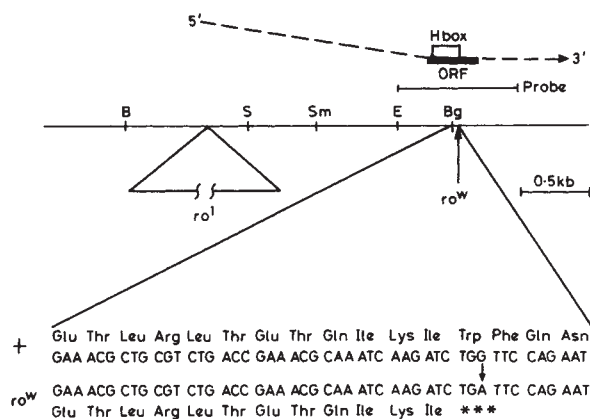


Fig. 3 Analysis of *rough* mutations localized to the vicinity of the homoeo-box open reading frame. The site of the open reading frame (ORF), the homoeo box (Hbox), site of insertion of a middle-repetitive DNA sequence in the *ro*¹ mutation and the site of sequence alteration in the *ro*^w mutation are indicated. The wild-type sequence (+) is given above the equivalent *ro*^w sequence. The arrow indicates the base change which creates a premature stop codon in the homoeo box region of *ro*^w. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; S, *Sal*I; Sm, *Sma*I.

Methods. A *D. melanogaster* stock, isochromosomal for the third chromosome, was prepared by mating a single male heterozygous for a marked third chromosome and a third chromosome balancer, TM3, to females carrying the TM3 chromosome. Flies heterozygous for the marked chromosome and TM3 were self-crossed to yield the isochromosomal stock. This population was exposed to EMS³⁶ and a *rough* mutant allele was isolated (W. R. Knibb and R.B.S., unpublished observations). Genomic DNA from the *ro*^w and *ro*¹ mutant chromosomes was cloned using an EMBL3 vector and sequenced using the dideoxy chain-termination method³⁵.

A DNA fragment was isolated from a library of Canton S *D. melanogaster* genomic DNA using, as probe, a synthetic 33-base oligonucleotide homologous to a consensus homoeo-box sequence. Hybridization *in situ* to salivary gland polytene chromosomes localized this DNA fragment to region 97D3-5 of the right arm of chromosome 3 (Fig. 1). Sequence analysis (Fig. 2) revealed an open reading frame encoding an amino-acid sequence with striking homology to the homoeo-box sequence, the highest homologies being 57% and 58% with the *Antennapedia* and *Sex combs reduced* homoeo-box sequences, respectively. One unusual feature is an arginine residue (residue 39 in Fig. 2b) at the centre of the proposed β -turn region of the 'helix-turn-helix' DNA binding domain²³. The presence of a residue with a large positively charged side-chain contrasts with much smaller, uncharged residues at the equivalent site in all other homoeo-box genes described. It is possible that the unusual charge on this residue is countered by a negatively charged amino acid elsewhere within the protein, allowing formation of the postulated β -turn at this site.

Molecular analysis of mutations mapping to the 97D region demonstrated that the exon encoding the novel homoeo box constitutes part of the *rough* (*ro*) locus. Restriction endonuclease and sequence analysis of DNA cloned from a *D. melanogaster* strain carrying the *ro*¹ mutation (results not shown) revealed the insertion of a middle-repetitive DNA sequence into the presumptive intron 5' to the homoeo box (Fig. 3). It should be noted, however, that there is no direct evidence that this insertion is the cause of the *ro* phenotype. As part of a genetic analysis of the 97D region, we isolated other mutant alleles by mutagenizing a strain of *D. melanogaster*, which had been made isochromosomal for the third chromosome, with ethyl methane sulphonate (EMS) (W. R. Knibb, R. G. Tearle, A.E. and R.S., unpublished observations). One EMS-induced allele of *rough*, *ro*^w, was shown

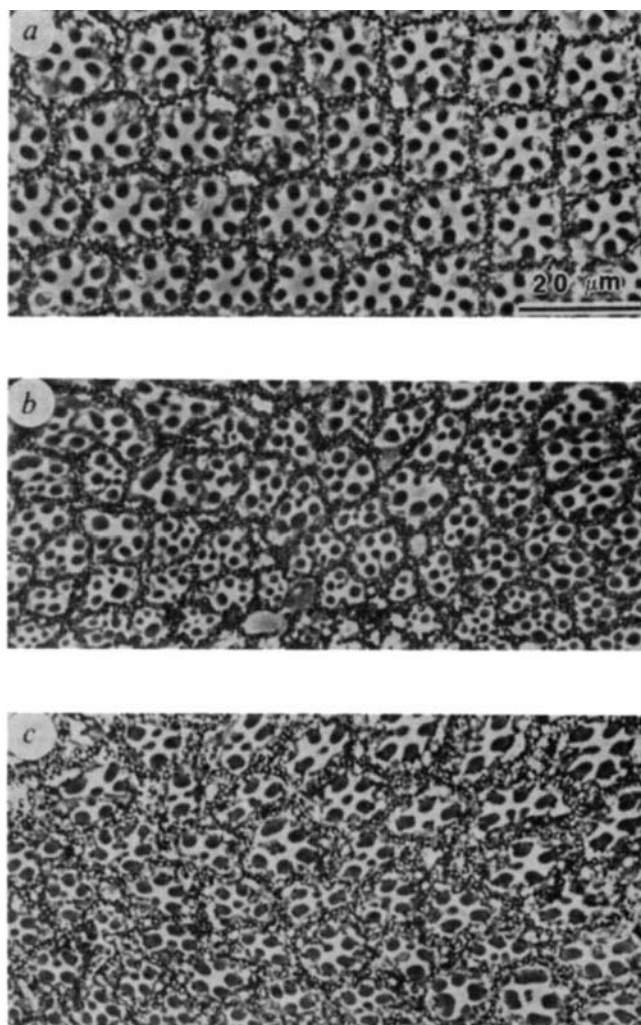


Fig. 4 Tangential sections of wild-type and *rough* mutant eyes showing the variable number of rhabdomeres and thus photoreceptor cells in the *rough* mutants. *a*, Wild-type with the characteristic arrangement of seven rhabdomeres. *b*, *ro*¹, variable number of rhabdomeres per ommatidium. *c*, The presumptive null allele, *ro*^w (see Fig. 3) shows similar but lesser variability to *ro*¹, with the majority of ommatidia having five or six rhabdomeres.

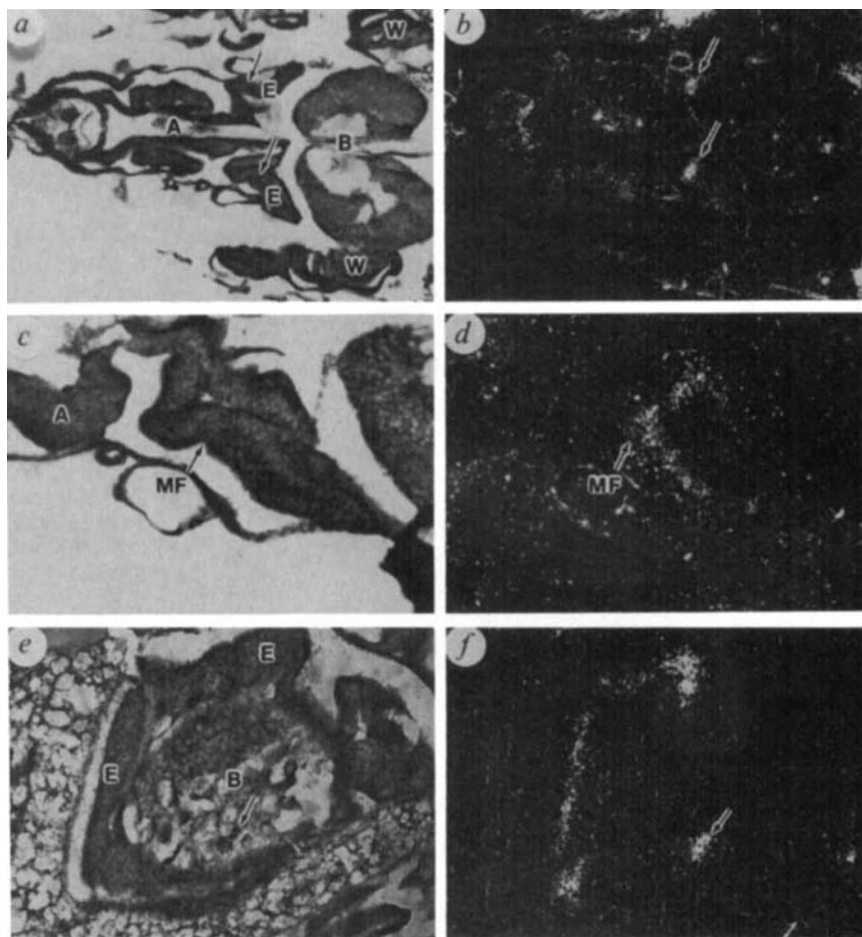
Methods. Heads of flies were cut off, sliced mid-sagittally and fixed immediately in 2% glutaraldehyde, 0.05 M cacodylate buffer pH 7.2 and 5% sucrose. The tissue was washed in buffer and postfixed in 1% OsO₄ in buffer, rehydrated with ethanol then embedded in Spurr's resin via propylene oxide. Sections (0.5 μ m) were stained with 1% toluidine blue in 1% borax, pH 8.9, and photographed.

by DNA sequence analysis to carry a single alteration in the parental nucleotide sequence which would lead to premature termination of translation within the homoeo box (Fig. 3). Further molecular characterization of the *rough* gene has been hampered by our inability to detect a corresponding complementary DNA clone in any available cDNA library, probably due to the restricted spatial and temporal expression of the gene (see below).

The *rough* locus of *D. melanogaster* is required for the proper differentiation of cells that comprise the ommatidia of the compound eye^{17,18}. Wild-type flies contain seven photoreceptor cells per ommatidium. Ommatidia in flies homozygous for *ro*¹ contain too few, or occasionally too many, photoreceptor cells (ref. 18 and Fig. 4). The presumptive null allele, *ro*^w, (Fig. 3) also exhibits variability in the number of photoreceptor cells per ommatidium,

Fig. 5 Localization of expression of the gene to third instar larval tissue sections. The region of the open reading frame indicated in Fig. 3 was used as probe. *a*, Bright-field micrograph of a section through a late third instar larva. *b*, Dark-field micrograph of the same section showing specific hybridization to the morphogenetic furrow of the eye portion (E) of the eye imaginal disc (arrowed). The wing disc (W) and the antennal portion (A) of the eye-antennal imaginal disc are not labelled. Non-specific binding of the probe to regions of cuticle can be seen in this section. *c*, Bright-field micrograph of a section through a late third instar eye-antenna imaginal disc. *d*, Dark-field micrograph of the same section showing hybridization in and posterior to the morphogenetic furrow (MF, arrowed), primarily in the apical regions of the tissue. *e*, Bright-field micrograph of a tangential section through the eye disc and brain of a third instar larva. *f*, Dark-field micrograph of the same section showing the characteristic hybridization to the morphogenetic furrow and apical regions posterior to it. In addition hybridization was observed to a region of the brain (B) of the third instar larva (arrowed). A, antennal imaginal disc; B, larval brain; E, eye imaginal disc; MF, morphogenetic furrow; W, wing imaginal disc.

Methods. Canton S wandering third instar larvae were collected then fixed, wax embedded and sectioned³⁷. Sections were hybridized with a ³⁵S-labelled single-stranded DNA probe³⁸ prepared from an M13mp19 clone of the fragment shown in Fig. 3. Slides were dipped in Kodak NTB2 photographic emulsion and exposed for 20–30 days.



characteristically having five or six, but this variability is less than in *ro*¹. The greater variability in the *ro*¹ ommatidial structure suggests that the *ro*¹ mutation does not completely inactivate the *rough* gene. In both mutants, this variation prevents the assembly of the normal crystalline array of ommatidia and results in the characteristic roughened appearance of the eye surface. Thus we would expect the *rough* gene to be expressed in the developing eye. This was confirmed using *in situ* hybridization to tissue sections. A probe covering the open reading frame (Fig. 3) was hybridized to tissue sections of third instar larvae. Low levels of expression were found to be restricted to the developing eye-antennal imaginal disc and a small region of the brain (Fig. 5). Expression in the imaginal disc was limited to that region destined to give rise to the retina and was maximal at the site of the morphogenetic furrow, although hybridization was also detected apically in more posterior regions through which the morphogenetic furrow had passed.

Expression of *rough* exhibits striking similarities with the expression of the *sevenless* gene, a gene required for correct formation of the seventh photoreceptor cell^{25–26}, which is expressed after passage of the morphogenetic furrow, predominantly in the apical regions of the tissue^{27–30}. Interestingly, the similarities may extend to expression in the brain. The *sevenless* gene product has been detected in the brain²⁹, but the spatial distribution has not been reported. Expression of *rough* in the larval brain was unexpected. The axon array which connects the retina to the optic lobe of the adult brain is disrupted in *rough* mutants, but clonal analysis has shown that this is dependent on the mutant genotype of the retina and not of the brain¹⁸. Expression of the *rough* gene in the larval brain may therefore have a function which has yet to be identified.

The site of maximal expression of the *rough* gene, the mor-

phogenetic furrow, is the site of the early pattern-forming events in retinal development. Cells in advance of the furrow appear undifferentiated, while cells through which the furrow has passed are in various stages of differentiation^{22–31}. Differentiation of cells in this tissue is not lineage dependent^{19,20} but appears to involve a form of 'recruitment' of individual cells into pre-clusters of photoreceptor cells²². The altered pattern of photoreceptor cells in *rough* mutants, and our finding that *rough* expression is strongest in the morphogenetic furrow of the eye disc, strongly suggests that this gene regulates aspects of the early pattern-forming processes in the developing eye.

It is not surprising that a new homoeo-box gene plays a role in pattern-forming events. It is significant, however, that pattern formation in the developing eye involves the assignment of different identities to individual cells, while pattern formation in early embryogenesis operates to define groups of cells in a metamer fashion (by genes such as *ftz*, *eve*, *prd*) or in specific anterior-posterior (by genes such as *Anip*, *Ubx*), or dorsal-ventral (by *zen* for example) positions. The expression of *rough* in a region of the eye-antennal disc satisfies the region-specific expression expected of a true homoeotic gene. The homoeotic gene *Deformed* is also expressed in the developing eye-antennal disc, but in the antennal portion of this disc^{32,33}. The effect of *rough* mutations, however, appears not to be that expected of a homoeotic transformation, as the basic structure of the tissue is not radically altered to resemble another region of the fly. A further difference between *rough* and other homoeo-box genes is suggested by our failure to find expression of this gene before the appearance of the morphogenetic furrow during the third larval instar. We have searched for expression in embryos and first and second instar larvae using *in situ* hybridization to tissue sections, northern blot analysis of isolated messenger RNA and

by screening of libraries of embryonic cDNA (results not shown). Although we cannot rule out low-level expression in a few cells, it appears that, unlike all other *Drosophila* homoeo-box genes described to date, *rough* is not expressed in early embryogenesis. The genetic evidence is consistent with these observations. Individuals homozygous for the *ro^w* allele, which we assume to be a null allele caused by a premature stop codon within the proposed DNA binding site of the homoeo box (Fig. 3), are fully viable and appear to undergo normal embryogenesis.

Thus the primary function of the *rough* gene appears to be markedly different to that of other *Drosophila* homoeo-box genes, with no apparent involvement in the generation of regional identities of groups of cells in early embryogenesis. The simplest hypothesis is that *rough* directly regulates the recruitment or differentiation of individual photoreceptor cells in the developing eye imaginal disc. This idea may not be as surprising as it seems. Many other *Drosophila* homoeo-box genes regulate imaginal development, albeit by processes that are less well understood than those occurring before and during the blastoderm stage. Furthermore, the syncytial nature of pre-blastoderm development in insects, onychophorans and myriapods is markedly different from the cellular nature of embryogenesis in the many other organisms in which homoeo-box genes are found. The involvement of *rough* in compound eye development may simply be a well defined example of a homoeo-box playing a regulatory role in pattern formation and differentiation in cellular tissues.

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The homologue of the Duchenne locus is defective in X-linked muscular dystrophy of dogs

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Duchenne muscular dystrophy (DMD) is the most common and the most severe of the muscular dystrophies in man¹. It is inherited as an X-linked recessive trait¹ and is characterized by ongoing necrosis of skeletal muscle fibres with regeneration and eventually fibrosis and fatty infiltration². Although the gene and gene product which are defective in DMD have recently been identified³⁻⁵, the pathogenesis of the disease is still poorly understood. A myopathy has been described in the dog⁶⁻⁸ which has been shown to be inherited as an X-linked trait⁹ and which is therefore a potential model of the human disease. We have studied the phenotypic expression of the disease, canine X-linked muscular dystrophy (CXMD), and have examined the molecular relationship between it and DMD. We report here that dogs with CXMD faithfully mimic the phenotype of Duchenne muscular dystrophy and that they lack the Duchenne gene transcript and its protein product, dystrophin.

Animals with CXMD were from a colony established by breeding an affected male with normal females to produce obligate carrier females⁹. Affected animals had early elevations of serum creatine kinase and developed clinical signs characterized by weakness and stiffness of gait by 8 to 10 weeks of age. Although clinical signs were always qualitatively similar and consistent within litters, their severity sometimes varied considerably between affected individuals in different litters. Severe myopathic changes were present in the muscle of dogs with the disease. There were many large hyaline, hypercontracted fibres, necrotic fibres infiltrated by macrophages, and abundant fibre regeneration (Fig. 1a). In some muscles evidence of endomysial and perimysial fibrosis was already present by four weeks of age (Fig. 1b). These changes were strikingly similar to those that have been reported in DMD in man².

To address the genetic relationship between CXMD and DMD, we carried out southern analysis of genomic DNA from dogs with CXMD in order to detect informative restriction fragment-length polymorphisms (RFLPs) or a defect in the canine homologue of the Duchenne locus. DNA extracted from cultured fibroblasts or tissues of normal, heterozygous and affected dogs was digested with various restriction endonucleases, and probed with several human DMD complementary DNAs. No evidence of a deletion or other defect in the coding portion of the Duchenne locus has so far been found, but informative RFLPs have been identified using human cDNA probes. In a preliminary linkage analysis with these markers

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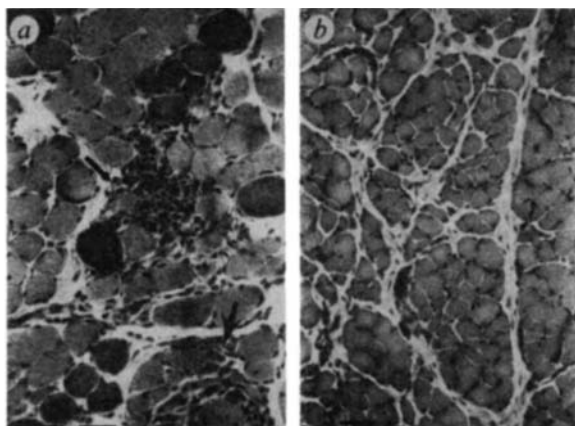


Fig. 1 *a*, Frozen section from the lateral head of the triceps muscle in a 4-month-old male dog with X-linked muscular dystrophy. Myopathic changes are characterized by numerous swollen dark staining, hypercontracted muscle fibres, macrophages infiltrating necrotic fibres (small arrow), and clusters of small regenerating fibres (large arrow). *b*, Frozen section from the cranial head of the sartorius muscle in a 4-week-old male dog with CXMD. There is marked perimysial and endomysial fibrosis.

Methods. Muscle biopsies were rapidly frozen in isopentane cooled in liquid nitrogen, sectioned at 8 μ m on a Reichert Jung Histostat cryostat, and stained with haematoxylin and eosin (*a*) or trichrome (*b*). Calibration bar, 50 μ m.

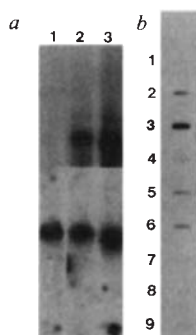


Fig. 2 *a*, Northern blot analysis of RNA from muscle of 4-week-old dogs and human control. Lane 1, affected dog; lane 2, control dog; lane 3, normal human control. Upper panel, hybridized with human DMD probes. In the control dog the DMD probe detected a 14-kb RNA comparable to that in the human control muscle. No signal was detected in the dystrophic dog muscle. Lower panel, hybridized with human fetal myosin heavy chain cDNA. The probe detected a 6.1-kb RNA with a signal in both control and dystrophic dogs comparable to that from human control muscle. *b*, Slot blot analysis of RNA from muscle of 4-week-old dogs. Slots 1-3, normal male; slots 4-6, obligate carrier female; slots 7-9, dystrophic male. Slots 1, 4 and 7 loaded with 1 μ g RNA, slots 2, 5 and 8 with 5 μ g and slots 3, 6 and 9 with 10 μ g. Minimal signal was detected in the affected dog. As there was no relationship to the amount of RNA applied, this signal was interpreted as background. The carrier female gave a signal of intermediate intensity.

Methods. Total muscle RNA was isolated using the guanidine isothiocyanate/caesium chloride gradient technique. For northern blots RNA was separated on a 1% agarose-formaldehyde gel, transferred to Biotrans nylon membrane (ICN) and hybridized at relaxed stringency with antisense DMD RNA labelled by *in vitro* transcription of pJK 1.4 (ref. 19) or with human embryonic myosin heavy chain cDNA labelled by primer extension. For slot blots, RNA was applied directly to nitrocellulose membrane under low vacuum and hybridized with a human cDNA³ corresponding to cDNAs 2 and 3 (ref. 4).

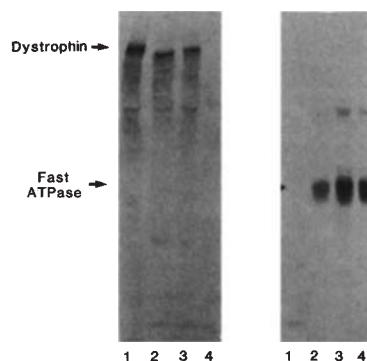


Fig. 3 Western blot analysis of total muscle protein (50 μ g). Shown are duplicate western blots with either dystrophin (first panel; 427K (ref. 20)) or a fast-twitch Ca^{2+} - Mg^{2+} ATPase isoform (second panel; 105K (ref. 21)) detected. Lanes: 1, normal mouse heart; 2, normal dog skeletal muscle; 3, CXMD carrier female skeletal muscle; 4, CXMD hemizygous (affected) male skeletal muscle. Dystrophin is undetectable in the affected dog. Though the dystrophin in dog appears smaller than that in mouse, this was not reproducible. The carrier female contains approximately half of normal levels of dystrophin, given the relative densities of ATPase staining. This was a consistent result in repeated blots. Mouse cardiac muscle ATPase does not react with the monoclonal antibody used.

Methods. Muscle samples from 4-week-old dogs were solubilized, electrophoretically fractionated (3.5-12.5% gradient SDS-polyacrylamide gels), transferred to nitrocellulose, and incubated with antibodies as previously described⁵. Dystrophin was detected using affinity purified sheep anti-mouse dystrophin raised against the '30K' antigen⁵. The fast twitch Ca^{2+} - Mg^{2+} ATPase isoform was detected using monoclonal antibody D2 (ref. 21). Immune complexes were visualized using alkaline phosphatase-conjugated second antibodies (Sigma).

there were no recombinations in eight meiotic events suggesting that the mutation responsible for CXMD is in or near the canine homologue of the Duchenne locus (data not shown).

To determine whether transcript for the DMD gene was expressed in dogs with CXMD, northern and slot blots were prepared¹⁰ and hybridized with the DMD probes (see legends for details). In northern blots (Fig. 2*a*) prepared from muscle of control dogs a transcript of 14 kilobases (kb), similar to that in human and mouse muscle¹¹, was detected. In contrast, no transcript could be demonstrated in dogs affected by CXMD. Slot blot experiments confirmed the results of northern blots and revealed a signal of intermediate intensity in a heterozygous female (Fig. 2*b*). As a control for non-specific destruction of messenger RNA similar blots were probed with a cDNA for human fetal myosin heavy chain. This probe recognized a 6.1-kb transcript in muscle from both control and CXMD dogs (Fig. 2*a*).

Western blots⁵ were carried out on extracts from muscle of the same dogs used for northern blotting in order to determine whether dystrophin, the gene product of the DMD locus, was also absent. In normal dogs, antibodies to dystrophin detected a protein with a relative molecular mass (M_r) of $\sim 400,000$ (400K), indistinguishable from that present in human and mouse muscle⁵ (Fig. 3). No dystrophin was detected in muscle from the affected dog (lane 4). Muscle from the heterozygous female gave a band of lower intensity than the control indicating that she had intermediate levels of dystrophin. Control antibodies directed against a fast isoform of Ca^{2+} - Mg^{2+} ATPase detected a 105K protein in all three dogs (Fig. 3).

These results suggest that CXMD will provide an excellent animal model for DMD in humans. The similarities between

the two diseases are striking. Both are characterized by an early onset, degenerative myopathy in which acutely injured, hypercontracted fibres are prominent, and in which progressive bouts of necrosis, phagocytosis, and regeneration result in marked endomysial and perimysial fibrosis. In dogs with CXMD, as in the great majority of DMD patients⁵, dystrophin, the protein product of the Duchenne locus, is absent. The absence of the DMD transcript is consistent with this finding. The lack of dystrophin and its mRNA is unlikely to be a secondary phenomenon, as the transcript for a myosin heavy chain, and the fast isoform of Ca^{2+} - Mg^{2+} ATPase, are normally abundant in the muscle of affected dogs. This conclusion is supported by the fact that dystrophin has been shown to be normally abundant in a variety of other neuromuscular diseases in man¹².

A murine mutant, *mdx*, is X-linked¹³ and has been shown to lack dystrophin⁵. The *mdx* mouse is therefore considered also to be an animal model for DMD. Although the recurring fibre necrosis and regeneration are similar in the *mdx* mouse and DMD, the *mdx* mouse develops little or no detectable weakness¹³⁻¹⁵. Thus the *mdx* mouse is a poor clinical model for DMD. The CXMD dog is clinically, pathologically and genetically an excellent model of DMD.

A significant unresolved question is why the same biochemical defect in muscle produces severe, progressive disease in man and the dog, yet is non-progressive in the mouse. Furthermore, in the affected dogs that we have studied, all of which are derived from a single male and all of which therefore presumably carry the same mutation, there can be clinically apparent variation in the severity of expression of the disease. It will be important to elucidate the basis for such variation, which may be relevant to an understanding of the clinical heterogeneity reported in Duchenne muscular dystrophy in man¹⁶⁻¹⁸. It suggests that, in addition to the nature of the mutation, other factors may affect the phenotypic expression of the disease. Both the *mdx* mouse and the canine model of X-linked muscular dystrophy will therefore be important in contributing to an understanding of the pathogenesis of Duchenne/Becker muscular dystrophy. Because of its striking clinicopathological similarities to DMD, the canine disease will be particularly useful in studying the pathogenesis of muscle injury and fibrosis, and for the development and testing of therapeutic approaches, including gene therapy.

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T-cell-specific deletion of T-cell receptor transgenes allows functional rearrangement of endogenous α - and β -genes

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In B cells the loci encoding immunoglobulin chains usually show allelic exclusion; a given B cell transcribes and translates only one productively rearranged allele of the heavy and light chain loci¹⁻³. This ensures that each B cell expresses only one antigen receptor. The loci encoding T-cell receptor (TCR) α - and β -genes may behave similarly⁴⁻⁶. We have previously reported that the expression of a transgenic TCR β -chain prevents functional and nonfunctional V_{β} rearrangements in the endogenous β -chain loci but not $D_{\beta}J_{\beta}$ rearrangements⁷. We have also been unable to detect the expression of the TCR γ -chain locus in thymocytes of these mice (unpublished observations). To study the mechanisms involved in forming a mature T-cell repertoire further, we have constructed mice expressing α - and β -TCR transgenes derived from a cytotoxic T-cell clone that is specific for the male antigen H-Y in the context of H-2D^b MHC molecules. Here we show that in these mice rearrangement of endogenous α -chain loci is also suppressed, although to a lesser extent than rearrangement of β -chain loci. In addition, in male $\alpha\beta$ TCR transgenic mice we observed T-cell clones which had deleted both transgenic α - and β -chain genes and expressed endogenous α - and β -chain TCR genes. These cells are presumably derived from rare thymocytes that leave the male thymus because their TCR no longer recognizes self antigen. The vast majority of CD4⁺8⁺ nonmature thymocytes expressing α - and β -transgenes are deleted in the male thymus⁸.

TCR α - (Uematsu *et al.*, manuscript in preparation) and β - (ref. 7) genes were isolated from the cytotoxic T-cell clone B6.2.16 specific for H-Y antigen in the context of H-2D^b MHC molecules. The genes were coinjected into pronuclei of fertilized eggs from C57Bl/6J $\times$ DBA/2J F₁ hybrids and Swiss albino mice. After transfer into foster mothers, offspring were screened for the presence of the two transgenes by Southern blot analysis of tail DNA as previously described⁷. Two doubly-transgenic founder mice (number 71 from B6D2F₁ hybrid and number 87 from albino mice) were crossed with strain C57L and further analysed. Both transgenes were co-transmitted through the germline, indicating that they had integrated together into a single chromosomal site. About four copies of the α -chain transgene and two copies of the β -chain transgene were detected in both mouse lines.

Both transgenes are transcribed in a tissue-specific fashion in lines Tg 71 and Tg 87. As shown in Fig. 1 for mouse line Tg 71, using a probe specific for the V_{α} sequence of the transgene, correctly-sized transcripts were found in male and female spleen cells but only at a much reduced level in some of the other tissues. Very low transcription of the α -chain transgene was observed in mitogen-stimulated B cells as compared with thymus (not shown), but no transcripts were detected with the $V_{\alpha 3}$ probe

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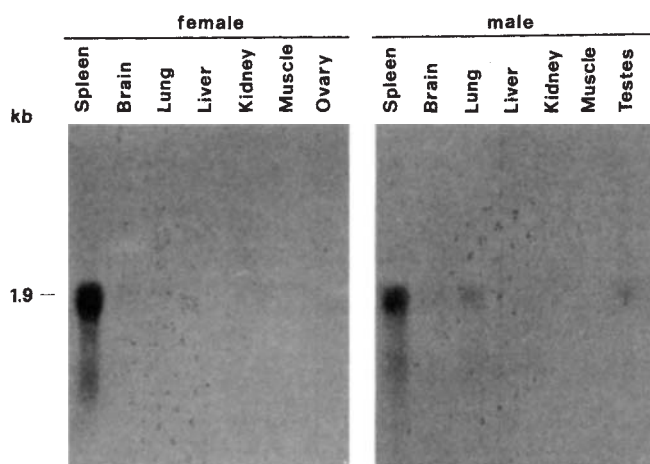


Fig. 1 Tissue-specific transcription of the α -chain transgene in female and male $\alpha\beta$ TCR transgenic mice. Northern blot hybridization of total RNA from tissues indicated with a $V_{\alpha 3}$ probe for the α -chain transgene.

Methods. Functionally rearranged α - and β -chain TCR genes were isolated from the cytotoxic T cell clone B6.2.16 specific for the male antigen H-Y and restricted by H-2D^b MHC molecules as described (ref. 7 and Y.U. *et al.*, manuscript in preparation). In the α -chain transgene, $V_{\alpha 3}$ is rearranged to $J_{\alpha 27}$ and the β -chain transgene is composed of $V_{\beta 8.2}$, $D_{\beta 1}$, $J_{\beta 2.3}$ and $C_{\beta 2}$. Inserts were excised from cosmid clones HY α 9.1-36 and HY β 9.1.14-5 and ~100 copies each were co-injected into pronuclei of fertilized eggs of either C57BL/6J $\times$ DBA/2J F₁ hybrids or Swiss Albino mice according to Hogan *et al.*¹¹. Microinjected eggs were implanted into oviducts of pseudopregnant Swiss Albino foster mothers and carried to term. Offspring were screened for integration of both transgenes by Southern blot hybridization of tail DNA with $V_{\alpha 3}$ and $J_{\beta 2}$ as described⁷. Transgenic offspring were crossed with strain C57L/J to establish $\alpha\beta$ TCR transgenic mouse lines. RNA was isolated from different organs of transgenic mice by the guanidinium/caesium chloride method as described¹². Total cellular RNA (10 μ g) was separated on 1.5% agarose-formaldehyde gels¹² and transferred to Zeta probe membranes (BioRad). Northern blots were hybridized with the $V_{\alpha 3}$ probe and exposed to Kodak X-Omat AR films for 8–48 h. The size of hybridizing RNA was determined by comparison with 18 and 28S ribosomal RNA. In a separate experiment quantitative Northern blot analysis was performed with spleen RNA from female and male transgenic mice using the $V_{\alpha 3}$ probe and subsequently an actin-specific probe as reference. The autoradiograms were analysed with an Ultrascan Laser Densitometer (LKB) and the intensity of the $V_{\alpha 3}$ -specific hybridization signal normalized for each lane with the actin signal.

in spleen cells of nontransgenic mice under the same hybridization conditions. Northern blot analysis using an actin-specific probe as reference indicated that transcription of the α -chain transgene was the same or possibly slightly less in male than in female mice (not shown). Previously we showed that transcription of the β -chain transgene is also T-cell specific and is dependent on a downstream regulatory sequence^{7,9}.

The majority of male and female T lymphocytes in line Tg 71 express the transgenic β -chain⁸. Of the CD8⁺ T lymphocytes from female transgenic mice, >30% respond specifically to male cells, indicating that the transgenic $\alpha\beta$ TCR is expressed in a large proportion of T cells. T lymphocytes from male transgenic mice, on the other hand, are tolerant to male target cells. One mechanism that establishes self-tolerance in male transgenic mice is the deletion of CD4⁺8⁺ immature thymocytes expressing α - and β -chain transgenes and high levels of CD8 molecules⁸.

Not all T cells in female transgenic mice, however, are male-specific. Figure 2 shows that both male and female transgenic mice contain T cells that can kill CBA cells but not syngeneic male target cells. The parental cytotoxic T cell clone B6.2.16 does not show this alloreactivity, indicating that at least some

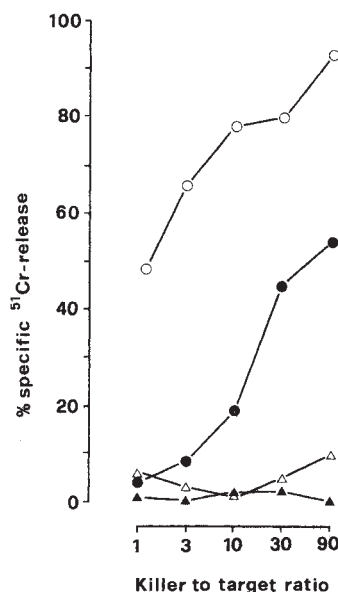


Fig. 2 Cytolytic activity of alloreactive T cells from $\alpha\beta$ TCR transgenic female (open symbols) and male (closed symbols) on CBA/J (O, ●) and C57BL/6J male (Δ, ▲) target cells.

Methods. Lymph node cells (5×10^6) were cultured at 37 °C in 5% CO₂ atmosphere together with 5×10^6 X-ray-irradiated (3,000 R) CBA/J spleen cells in 2 ml of Iscove's modified Dulbecco medium supplemented with 5% IL-2. After 5 days cells were collected and assayed for cytolytic activity on ⁵¹Cr-labelled lymphoblasts as described¹³.

T cells in male and female transgenic mice have rearranged endogenous T-cell receptor genes or, less probably, mutated the transgenes. Rearrangement of endogenous α -chain genes was monitored via deletion of C_{δ} sequences and rearrangement of endogenous β -chain genes with $D_{\beta 1}$, $D_{\beta 2}$, $J_{\beta 2}$ and C_{β} -specific probes. As shown in Fig. 3, and summarized in Table 1, two of six independent H-Y-reactive T-cell clones from female mice contained at least one endogenous α -chain allele in germline configuration. The other four clones had rearranged both α -chain alleles. None of them, however, showed a VDJ rearrangement of the β -chain locus. Two alloreactive T-cell clones from female transgenic mice showed rearrangement of both endogenous α -chain alleles and again no VDJ rearrangement of the β -chain locus. All clones transcribed the transgenic α - and β -chain genes and expressed the β -chain transgene. These data indicate that expression of the β -chain transgene exerts allelic exclusion of the endogenous β -chain genes as previously described⁷, whereas expression of the α -chain transgene suppresses expression of endogenous α -chain genes incompletely. Thus it appears that a T-cell receptor composed of an endogenous α - and a transgenic β -chain ($\alpha_E\beta_T$) is responsible for the alloreactivity. We do not know whether the alloreactive T cells in female transgenic mice express two different receptors on their surface, namely $\alpha_T\beta_T$ and $\alpha_E\beta_T$. If so, the amount of $\alpha_T\beta_T$ must be low because CD8⁺ T cells do not kill male targets (Fig. 2).

T cells in male transgenic mice are largely CD4⁺8⁺ (ref. 8). Mitogenic stimulation of male CD4⁺8⁺ T lymphocytes with Con A and subsequent Southern blot analysis of the uncloned population revealed that at least 50% of the endogenous α - and β -chain loci are in germline configuration (Fig. 3 and Table 1). These cells express the transgenic β -chain and transcribe the α -chain transgene, indicating that they express the transgenic T-cell receptor, although they do not respond to male stimulator cells⁸.

Analysis of three alloreactive T-cell clones from male transgenic mice reveals a completely different picture. In all three clones both endogenous α -chain alleles were rearranged and

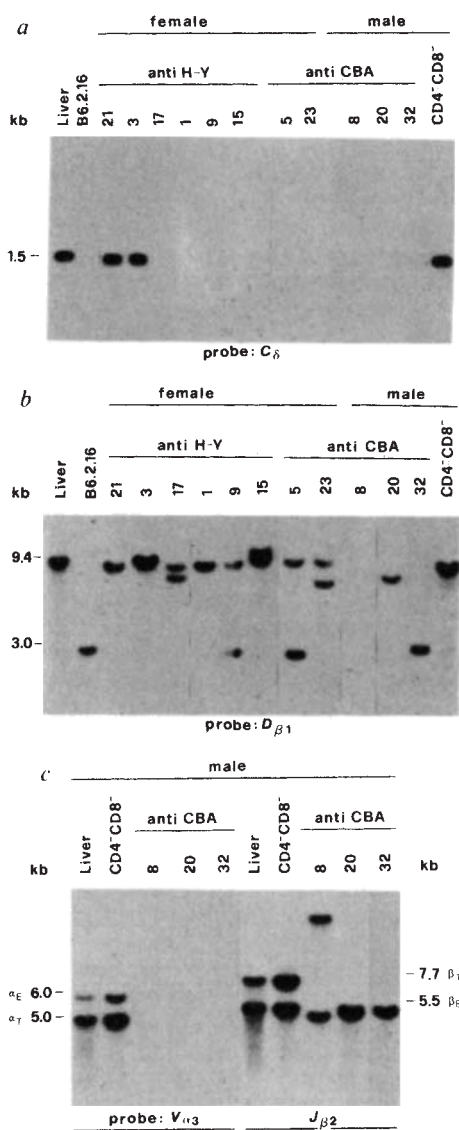


Fig. 3 Southern blot analysis of male-reactive and alloreactive T-cell clones and lines from $\alpha\beta$ TCR transgenic mice. *a*, DNA (10 μ g) from cells indicated was digested with *EcoRI* and hybridized with a C_δ probe. From the female transgenic mouse, two of the six male-reactive T-cell clones contained at least one endogenous α -chain allele in germline configuration as shown by the presence of the C_δ gene segment. The other four clones had rearranged both α -chain alleles. The same is true for alloreactive T-cell clones from the female as well as from the male transgenic mouse. CD4⁺CD8⁺ T cells from the male, however, contain their endogenous α -chain genes predominantly in an unrearranged configuration. DNA from transgenic liver and TCR donor clone B6.2.16 was used as control. *b*, *HindIII*-digested T-cell DNA was hybridized with a $D_{\beta 1}$ -specific probe detecting 5' flanking sequences. The 9.4-kilobase (kb) band represents the germline configuration, smaller-sized bands $D_{\beta 1}$ to $J_{\beta 1}$ or $D_{\beta 1}$ to $J_{\beta 2}$ rearrangements. Joining of V_β to any partial D_β - J_β rearrangement will result in a deletion of the sequence detected by the $D_{\beta 1}$ probe. Together with successive hybridizations of the same filters with $D_{\beta 2}$, $J_{\beta 2}$ and C_β -specific probes, the results indicate that there are partial β -chain gene rearrangements in male-reactive as well as alloreactive T-cell clones from the female transgenic mouse, whereas there are complete *VDJ* rearrangements on at least one allele in each alloreactive T-cell clone from the male. At least 50% of CD4⁺CD8⁺ T cells from the male have kept their β -chain genes unrearranged. DNA from transgenic liver and clone B6.2.16 served as control. *c*, DNA from alloreactive male T-cell clones was double-digested with *PvuII* and *SalI* and hybridized successively with $V_{\alpha 3}$ and $J_{\beta 2}$ probes. With the $V_{\alpha 3}$ probe neither transgenic nor endogenous $V_{\alpha 3}$ sequences could be detected in the three T-cell clones. The $J_{\beta 2}$ probe hybridized only to endogenous β -chain gene segments. Hybridization of *HindIII*-digested DNA from the same clones with a C_α probe also revealed only endogenous α -chain genes (not shown). CD4⁺CD8⁺ T cells, however, still contained both transgenes. This indicates that both transgenes were deleted in alloreactive T-cell clones from the male as were endogenous $V_{\alpha 3}$ segments. DNA from transgenic liver was included for comparison.

Methods. T-cell clones were established from female and male transgenic mice by stimulation with irradiated male C57Bl/6 or allogeneic CBA spleen cells as described¹³. F23.1⁺ CD4⁺CD8⁺ T cells were isolated from male transgenic mice by treating lymph-node cells with a mixture of cytotoxic anti-CD4 (RL172, ref. 14) and anti-CD8 (MO2.2, ref. 15) monoclonal antibodies plus rabbit complement as described¹⁶. Viable cells were recovered by centrifugation through Ficoll, stained with biotinylated F23.1 antibody plus phycoerythrin-streptavidin and sorted with a fluorescence-activated cell sorter FACS 440 (Becton Dickinson). Sorted F23.1⁺ cells (>99% pure 1×10^6 ml⁻¹) were stimulated for 4 days with concanavalin A (1 μ g ml⁻¹) in the presence of IL-2. DNA and RNA were isolated from T cells by differential centrifugation¹². Banded DNA was removed from the gradient, precipitated with ethanol after fivefold dilution with distilled water and phenol-chloroform extracted¹². For analysis of endogenous α -chain gene rearrangements, 10 μ g of DNA was fractionated on 1.5% agarose gels after *EcoRI* restriction and transferred to Zeta probe membranes¹⁷. Southern blots were hybridized with a 0.8-kb *MboII*-*EcoRI* fragment containing C_δ sequences¹⁸ as described¹⁹. Rearrangements of endogenous β -chain genes were analysed with *HindIII*-digested DNA and $D_{\beta 1}$, $D_{\beta 2}$, $J_{\beta 2}$ and C_β probes⁷. Deletion of transgenes was detected after double digestion of DNA with *PvuII* and *SalI* and sequential hybridization of the filter with $V_{\alpha 3}$ (see legend to Fig. 1) and $J_{\beta 2}$ probes.

Table 1 Characteristics of male-reactive and alloreactive T cell clones and lines from female and male transgenic mice

Origin	T cell Specificity	Clone	Rearrangement of endogenous alleles		Transgene		Phenotype		
			α_E^*	$\beta_E^\dagger$	Transcription $\alpha_T^\ddagger$	Expression $\beta_T^\S$	CD8 §	CD4 §	
Transgenic female	anti H-Y	21	—	g/g	+	+	+	—	
		3	—	DJ/DJ	+	+	+	+	
		17	+	g/DJ	+	+	+	—	
		1	+	DJ/DJ	+	+	+	—	
		9	+	DJ/DJ	+	+	+	—	
		15	+	DJ/DJ	+	+	+	—	
	anti CBA	5	+	g/DJ	+	+	+	—	
		23	+	DJ/DJ	+	+	+	—	
	Transgenic male	ConA stimulated CD4 ⁺ 8 ⁺ T cells		—	g/g	+	+	—	—
		anti-CBA	8	+	VDJ/VDJ	—	—	+	—
20			+	VDJ/DJ	—	—	+	—	
32			+	VDJ/DJ	—	—	+	—	

* Determined by Southern blot analysis with the C_δ probe. Dash indicates that C_δ sequences are present on at least one of the two alleles; +, C_δ sequences are deleted on both alleles.

† Southern blot analysis with $D_{\beta 1}$, $D_{\beta 2}$, $J_{\beta 2}$ and C_β probes.

‡ Northern blot analysis with the $V_{\alpha 3}$ probe.

§ Determined by cytofluorimetric analysis with a FACScan (Becton Dickinson) using monoclonal antibodies specific for $V_{\beta 8}$, CD8 and CD4 as described previously⁸.

|| Comparison of liver and uncloned CD4⁺CD8⁺ T-cell DNA by Southern blot hybridization indicates that α_E and β_E alleles are predominantly in germline configuration.

also at least one of the two endogenous β -chain alleles showed VDJ rearrangement. But no transcription of the transgenic α - and β -chain genes and no expression of the β -chain transgene was detected. Surprisingly, Southern blot analyses of these T-cell clones with specific probes revealed a complete deletion of the transgenic α - and β -chain sequences (Fig. 3 and Table 1). Because these cells are not male-specific and do not express the transgenic receptor they express normal levels of CD8. Cells with normal levels of CD8 were indeed detected in low numbers in $\alpha\beta$ TCR transgenic males⁸.

T lymphocytes in male transgenic mice are therefore heterogeneous with respect to the presence and expression of the transgenes. Although most T lymphocytes contain and express the transgenes, some T lymphocytes have lost them, possibly by unequal sister chromatid exchange. Short stretches of sequences are usually duplicated at the integration site¹⁰ and it is conceivable that homologous recombination between these repeats has excised the integrate. We assume that this deletion is a rare event, occurring early in T-cell development, which removes the block otherwise exerted on the rearrangement of endogenous α - and β -chain genes. Because some of these cells are not autosppecific they are not deleted and can accumulate in male mice.

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The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4

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CD4, a cell-surface glycoprotein expressed on a subset of T-cells and macrophages, serves as the receptor for the human immunodeficiency virus (HIV) (reviewed in ref. 1), binding to the HIV envelope glycoprotein, gp120 with high affinity^{2,3}. Attempts to block infection *in vivo* by raising antibodies against gp120 have failed, probably because these antibodies have insufficient neutralizing activity⁴. In addition, because of the extensive polymorphism of gp120 in different isolates of HIV, antibodies raised against one HIV isolate are only weakly effective against others⁵. Because interaction with CD4 is essential for infectivity by all isolates of HIV, an agent that could mimic CD4 in its ability to bind to gp120, such as a peptide or monoclonal antibody, might block infection by a wide spectrum of isolates. To aid the identification of such a ligand we have defined regions of CD4 that are required for binding to gp120. Although human CD4 is similar to mouse CD4 in amino-acid sequence (55% identity, ref. 6) and structure^{7,8}, we have found that the murine protein fails to bind detectably to gp120 and have exploited this finding to study binding of gp120 to mouse-human chimaeric CD4 molecules. These studies show that amino-acid residues within the amino-terminal immunoglobulin-like domain of human CD4 are involved in binding to gp120 as well as to many anti-CD4 monoclonal antibodies.

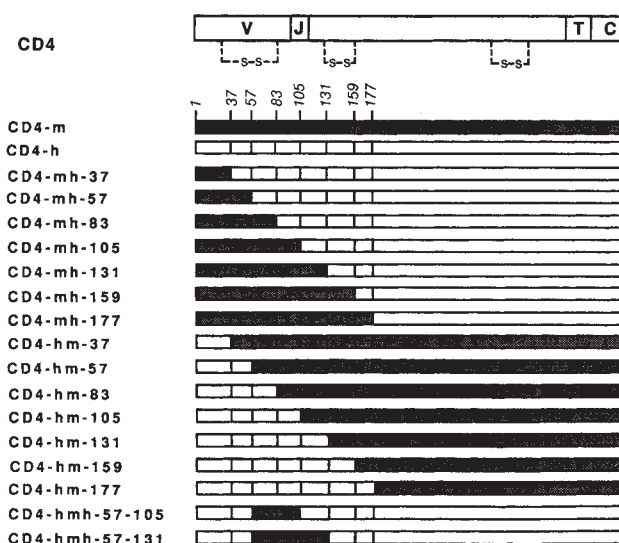
To compare the relative abilities of mouse and human CD4 to bind to gp120, Chinese hamster ovary (CHO) cell lines were transfected with a vector expressing either mouse or human CD4 (see legend, Fig. 2a). Expression of CD4 at the cell surface was investigated by cytofluorometric analysis of the cell lines stained with anti-mouse or anti-human CD4 monoclonal antibody (MAbs YTA-312 or OKT4, respectively) plus fluorescein-conjugated goat anti-mouse immunoglobulin (FITC-GAMlg). The relative gp120 binding ability of the CD4 molecules was

assessed in a similar assay, incubating the cells sequentially with recombinant gp120, monoclonal anti-gp120 antibody 110-3, and FITC-GAMlg. The cell line transfected with the human CD4 expression vector showed a high level of fluorescence with OKT4 and a similar high level with gp120 plus anti-gp120 (Fig. 2a). In contrast, the cell line transfected with the mouse CD4 expression vector showed a high level of fluorescence with YTA-312 (corresponding to a level of CD4 expression ~10-fold greater than that of mouse T-cell tumour lines) but no significant increase over background fluorescence with gp120 plus anti-gp120. Based on this result, and on additional results with CHO cells expressing highly amplified mouse CD4 genes, the minimum difference in gp120-binding capacity between mouse and human CD4 is ~1,000-fold.

Although CD4 consists of four external domains, a soluble form of human CD4 that contains only the first two domains (amino-acid residues 1-177) binds effectively to gp120 (ref. 9 and A. Moriarty and D.R.L., unpublished results). In addition, a chimaeric cell-surface molecule containing the first two domains of human CD4, linked to the membrane anchor region of human CD8, functions as an HIV receptor¹⁰. We therefore focused our attention on the first two domains of CD4 and prepared chimaeric CD4 cDNAs in which the sequence encoding the first two domains of the protein was made up of (1) murine sequence followed by human sequence, (2) human sequence followed by murine sequence and (3) human sequence followed first by murine sequence and then by more human sequence (CD4-mh, CD4-hm and CD4-hmh, respectively, see Fig. 1). The chimaeric cDNAs had no insertions or deletions of amino-acid residues.

To compare the relative gp120 binding abilities of the CD4 molecules encoded by the chimaeric cDNAs, we established CHO cell lines expressing these chimaeric proteins. Staining of the CD4-hm and CD4-hmh-expressing cell lines with anti-mouse CD4 mAb YTA-312 or anti-human CD4 MAb OKT4, respectively, showed a broad profile of CD4 expression (Fig. 2b). CD4-mh-expressing cells were stained with OKT4 and showed similar results (both antibodies recognize membrane-proximal regions of CD4; N.R.L., unpublished data). The heterogeneity of CD4 expression is probably due to variability in the extent of amplification of the transfected CD4 genes; however, the majority of the cell lines express CD4 at levels higher than those of T-cell tumour lines. Misfolding of the chimaeric CD4 proteins is a possibility that cannot be excluded but does not seem likely

Fig. 1 Structure of chimaeric CD4 cDNAs. Restriction sites were created at the analogous positions of the mouse⁶ and human¹⁷ cDNA clones using oligonucleotide-directed mutagenesis¹⁸. The mutations did not alter the encoded amino-acid sequence. New restriction endonuclease sites and positions were as follows: *Avr*II, 37; *Xba*I, 57; *Nde*I, 83; *Kpn*I, 105; *Bgl*II, 131; *Apa*LI, 159; *Sca*I, 177. (Amino-acid numbering differs by two amino acids from that previously reported¹⁷ in which the correct N-terminal residue of the mature protein is at position two.) A segment of each mutated CD4 cDNA was removed by cleavage at the new restriction site and a second site within the plasmid sequence and was replaced by the reciprocal mouse or human fragment. The structure of each chimaeric cDNA was confirmed by restriction mapping with enzymes diagnostic of human or mouse CD4 cDNA and, in several cases, by DNA sequence analysis of the junction region. These were then cloned into the expression vector pSV7d (derived from pHS210 by deletion of the gB gene, ref. 19). pSV7d contains the simian virus-40 origin of replication, early region promoter and polyadenylation sequence. S-S denotes intra-chain disulphide bonds; V, region homologous to immunoglobulin variable region; J, region homologous to immunoglobulin J region; T, transmembrane domain; C, cytoplasmic domain. Grey bars denote mouse CD4 sequence and white bars denote human CD4 sequence.



because they are expressed at the cell surface¹¹ and are bound by monoclonal antibodies that recognize conformational determinants of CD4¹².

CHO cells expressing the chimaeric CD4 proteins were tested for their ability to bind gp120 (Fig. 2b and Table 1). Of the cell lines expressing chimaeric CD4 that contained amino-terminal murine sequence, only CD4-mh-37 bound gp120 (Table 1). This indicates that amino acids specific to human CD4 in the region of amino acids 1-37 are not necessary for gp120 binding, whereas amino acids 38-57 are indispensable. Chimaeras that contained human amino-terminal sequence displayed a more complex pattern of gp120 binding (Fig. 2b). Cells expressing CD4-hm-131, CD4-hm-159 and CD4-hm-177 showed full or near-full binding to gp120, whereas cells expressing CD4-hm-83 showed low-level binding and cells expressing CD4-hm-37 and CD4-hm-57 did not bind detectable amounts of gp120. These results suggest that amino-acid residues 1-83 of human CD4 are sufficient to bind gp120. Consistent with these results, cells expressing CD4-hmh-57-105 and CD4-hmh-57-131 failed to bind gp120, indicating a requirement for human sequence 57-105. Taken together, these data suggest that the minimum region of human CD4 necessary for binding of gp120 lies in the region between amino acids 37 and 83, and, in addition, that amino-acid residues 83-159 contribute to full gp120 binding activity. It is possible that amino acids 38-83 contain the entire gp120 binding

site but that the presence of murine-derived amino-acid sequence C-terminal to amino acid 83 in CD4-hm-83 results in a protein whose conformation interferes with gp120 binding.

In contrast to cells expressing CD4-hm-83, which bound a small amount of gp120, those expressing CD4-hm-105 did not detectably bind gp120. This result was unexpected because CD4-hm-105 contains more human-derived amino-acid sequence than does CD4-hm-83. It is unlikely that the protein encoded by CD4-hm-105 is misfolded, because nine monoclonal antibodies specific for the first external domain of CD4 bound fully to this chimaeric CD4 molecule (Table 1). It is possible, however, that amino acids 83-105, which link the first and second domains of the CD4 protein, might determine their relative orientation. In CD4-hm-105, human sequence 83-105 followed by mouse sequence 105-131 may result in a protein whose second domain hinders gp120 binding to the first CD4 domain. This hypothesis is supported by the antibody binding data (discussed below) which suggest close proximity of the two domains. Because of this conformational effect it could not be determined whether the two regions (83-105 and 105-131) form part of the gp120 binding site.

The majority of anti-CD4 monoclonal antibodies interfere with HIV infectability and a panel of such antibodies was tested for ability to bind to the chimaeric CD4 proteins (Table 1, Fig. 3). The results of these studies can be summarized as follows.

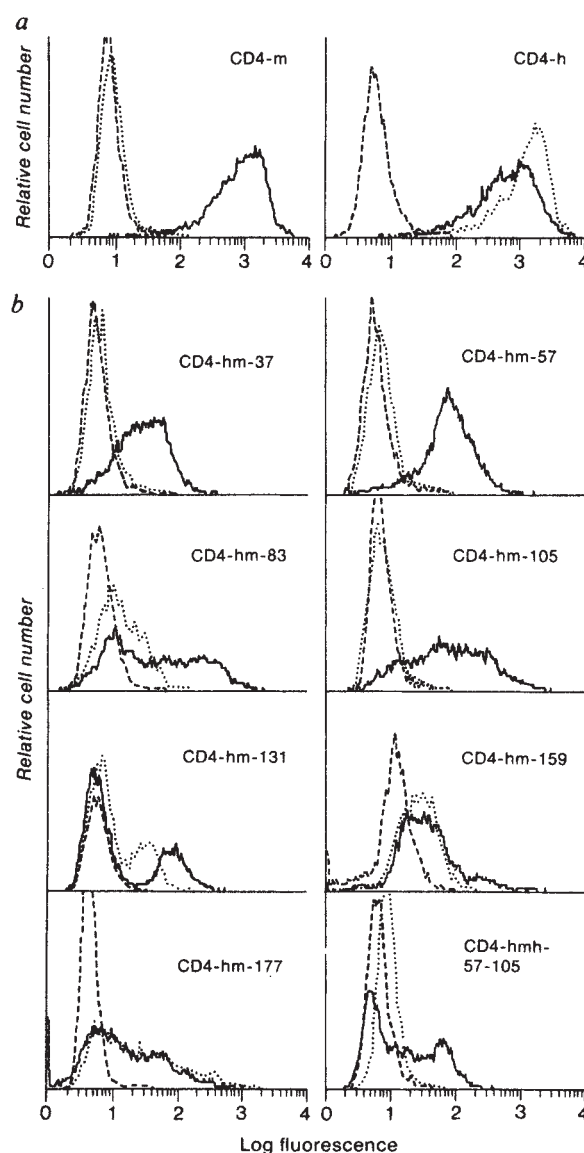
Table 1 Summary of gp120 and anti-CD4 monoclonal antibody binding to cells expressing chimaeric CD4

Chimaera	gp120	leu3a	OKT4a	OKT4b	OKT4c	OKT4e	OKT4f	MT151	MT321	VIT4	BU264	G19-2	66.1	13B.8.2	MAb1	G17.2
CD4-m	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND
CD4-h	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CD4-mh-37	+++	+++	+++	+++	+++	++	+++	+++	-	+++	+++	++	+	-	+++	+++
CD4-mh-57	-	-	+++	+++	-	-	-	-	-	++	+	+	-	-	-	-
CD4-mh-83	-	-	-	+++	-	-	-	-	-	+++	+	+	-	-	-	-
CD4-mh-105	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	ND
CD4-mh131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND
CD4-mh159	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CD4-mh-177	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND
CD4-hm-37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND
CD4-hm-57	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD4-hm-83	+	++	++	-	-	-	+	-	-	-	-	-	+	-	+	+
CD4-hm-105	-	+	++	-	-	-	-	-	-	+++	-	+	+	+++	+++	+
CD4-hm-131	++	+++	+++	+++	+++	+++	+++	+	++	+++	++	++	++	+++	+++	+++
CD4-hm-159	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	ND
CD4-hm-177	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	ND
CD4-hmh-57-105	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	ND
CD4-hmh-57-131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND

Gp120 and antibody binding levels were determined for each cell line as described in Fig. 2 and fluorescence intensity was compared to that obtained on staining with OKT4 or YTA-312: +++, 75-100 % of the fluorescence intensity of OKT4 or YTA-312; ++, 10-75 %; +, 2-10 %; -, <2 %. MAb1 is a group of antibodies that showed an identical pattern of binding to each of the cell lines. It includes MT310, BW264/123, F101-5, f101-69(ST4), T4/18T3AG, T419THY5DY, 91D6.

Fig. 2 Binding of gp120 and anti-CD4 monoclonal antibody to cells expressing mouse or human CD4 (*a*) and to cells expressing chimaeric CD4 (*b*). Cells were incubated with gp120 plus anti-gp120 antibody (dotted line), anti-gp120 antibody alone (dashed line), or anti-CD4 antibody (solid line). Control, untransfected CHO cells showed no increase over background level of fluorescence with either antibody (data not shown). Gp120 was a recombinant form of the protein²⁰ that contains a replacement of the 30 amino-terminal residues with 25 residues from the herpes simplex virus gD protein and an addition at the carboxy-terminus of 20 residues from the amino-terminus of HIV gp41.

Methods. The CHO DHFR⁻ (dihydrofolate reductase) cell line DXB11 was transfected by the calcium-phosphate method²¹ with CD4 expression vector DNA plus the DHFR expression plasmid, pMG1P (ref. 22). Surviving colonies were selected for amplification of the transfected genes by growth in increasing concentrations of methotrexate²³. Cells were tested for expression of CD4 and for their ability to bind gp120 as follows: 0.5×10^6 cells were incubated with YTA-312 or OKT4 (1 μ l ascites) or with recombinant gp120 (1 μ g) in 100 μ l phosphate buffered saline containing 1% fetal calf serum (PBS-FCS) for 30 min at 0 °C. The gp120-treated cells were washed, resuspended in 100 μ l PBS-FCS containing 200 ng anti-gp120 MAb 110-3 (ref. 24), and incubated for 30 min at 0 °C. All cells were then washed in 2.0 ml PBS-FCS, incubated in 100 μ l PBS-FCS containing 4 μ l FITC-GAM1g (Becton-Dickinson) for 30 min at 0 °C, washed again and resuspended in 0.5 ml PBS-FCS containing 0.2 μ g ml⁻¹ propidium iodide. Cell-surface fluorescence was analysed on a FACSCAN (Becton-Dickinson) and nonviable cells were removed by virtue of high propidium iodide fluorescence. Anti-mouse CD4 MAb YTA-312 was used on CD4-m and CD4-hm cell lines. Anti-human CD4 MAb OKT4 was used on CD4-h, CD4-mh and CD4-hmh cell lines. Both antibodies recognize regions of CD4 that lie carboxy-terminal to amino acid 198 (data not shown) and were therefore used to determine expression levels of chimaeric CD4 cell-surface protein.



(1) All but two (OKT4 and OKT4B) of the 22 antibodies tested require human sequence in the first domain of CD4. This first domain therefore appears to be highly antigenic compared to the rest of the protein, a feature that cannot be attributed to clustering of amino-acid sequence differences between mouse and human CD4 in this region as these are spread throughout the external domains of the protein. (2) Nearly all of the antibodies require regions of CD4 that overlap with the region involved in gp120 binding (amino acids 38–131). (3) The pattern of binding of many of the antibodies (for example MAb1, Leu3a, OKT4a, OKT4f, G17.2, 66.1) to the chimaeric CD4 proteins is similar to that of gp120; they require amino acids 38–105, show low-level binding to the chimaera containing human sequence 1–83 (CD4-hm-83) and no requirement for human sequence 1–37. (4) Unlike gp120, several antibodies (such as MAb1) bind CD4-hm-105. These antibodies do not require human sequence 105–131 and do not show the conformational effect previously observed in the lack of gp120 binding to this chimaera. (5) Several antibodies require human amino-acid residues that lie in both the first and second domains of CD4 (for example BU264, MT151, MT321), suggesting that the two domains interact and may be folded close to one another.

The antibodies whose pattern of binding to the CD4 chimaeras most resembles that of gp120 are Leu3a, OKT4f and G17.2. Leu3a binding has been shown to be strongly conserved in primate species whose cells can be infected with HIV (OKT4f

and G17.2 were not studied, ref. 13). In addition, Leu3a and OKT4f bind more effectively to CD4-hm-83 than to CD4-hm-105, indicating a conformational effect similar to that shown by gp120. These antibodies may, therefore, recognize CD4 amino-acid residues identical to those recognized by gp120 and might possess binding sites with similarity to the gp120 binding site for CD4 (ref. 3).

The binding pattern of antibodies OKT4b and VIT4 is consistent with localization of the minimum gp120 binding site to amino-acid residues 38–83. It has been shown that VIT4 blocks HIV infection completely¹⁴ whereas OKT4b blocks infection and gp120 binding incompletely¹⁵. As shown in Fig. 3, VIT4 appears to bind CD4 between amino acids 83–105, adjacent to the minimum gp120 binding site, whereas OKT4b appears to bind between amino acids 105–131, further from the minimum gp120 binding site but within the region required for full binding.

The anti-CD4 antibodies OKT4, G19.2 and 66.1 have been reported not to block HIV infection¹⁴. OKT4 binds to amino-acid residues outside the gp120 binding site (C-terminal to amino acid 196, data not shown). G19.2 requires human sequence close to that required by gp120 (amino acids 83–105, Fig. 3) even though it does not inhibit infection. This antibody may therefore contact amino acids near, but not within, the region of CD4 required for gp120 binding. The binding site of 66.1 appears to lie within the region required for gp120 binding; however, our

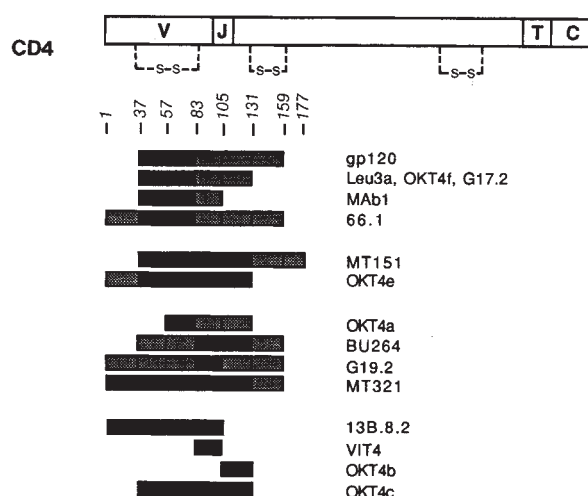


Fig. 3 Human CD4 regions required for gp120 and anti-CD4 monoclonal antibody binding. Results are derived from data in Table 1. Solid bars indicate the minimal human CD4-derived region required for significant binding; shaded bars indicate minimal human CD4-derived region required for full binding. MAb 1 represents the seven anti-CD4 monoclonal antibodies identified in the legend to Table 1.

results show that it competes effectively with gp120 for CD4 binding (data not shown).

In summary, the first domain of CD4 appears to be the region of the protein most available for interaction with other proteins as anti-CD4 antibodies and HIV both recognize this region. The second domain appears to interact with the first domain and is likely to be positioned near it. Gp120 appears to bind the portion of the first domain of CD4 that encompasses the region homologous to the second complementarity-determining region of immunoglobulin variable regions and may, in addition, bind residues of the second domain. The potential involvement of discontinuous regions of CD4 in gp120 binding makes it less likely that a peptide derived from a single region of CD4 would bind gp120 with high affinity, but peptides derived from amino acids 37–83 would be the best candidates. Several anti-CD4 antibodies (Leu3a, G19.2 and OKT4f in particular) bind to CD4 with sequence requirements similar to those of gp120. These antibodies may have antigen combining sites similar to the gp120 binding site for CD4 and therefore could be candidates for anti-idiotypic mimicry studies¹⁶.

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Internalization of the human immunodeficiency virus does not require the cytoplasmic domain of CD4

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Binding of the human immunodeficiency virus (HIV) to infectable host cells, such as B and T lymphocytes, monocytes and colorectal cells, is mediated by a high-affinity interaction between the gp120 component of the viral envelope glycoprotein and the CD4 receptor^{1–5}. Upon binding, it is thought that the second component of the envelope, gp41, mediates fusion between the viral envelope and host cell membranes^{6,7}. However, the early steps of HIV infection have not yet been thoroughly elucidated. Viral entry was first reported to be mediated by pH-dependent receptor-mediated endocytosis⁸; subsequent studies have shown entry to be pH-independent^{9,10}. Although direct fusion of virus to plasma membranes of infected cells has been observed by electron microscopy⁹, it is still formally possible that the infectious path of the virus involves receptor-mediated endocytosis. To gain a better understanding of receptor function in viral entry, we have analysed the ability of several altered or truncated forms of CD4 to serve as effective viral receptors. Our results indicate that domains beyond the HIV-binding region of CD4 are not required for viral infection. Some of the altered forms of CD4 that serve as effective HIV receptors are severely impaired in their ability to be endocytosed. These experiments therefore support the notion that viral fusion to the plasma membrane is sufficient for infection.

Site-directed mutagenesis was used to prepare a variety of CD4 mutants (see Fig. 1). CD4(glyc) lacks the two N-linked glycosylation sites found in wild-type CD4; CD4(cyt399) and CD4(401) have severely truncated cytoplasmic domains; and in CD4(ala408) and CD4(leu415), the cytoplasmic serine residues have been replaced. Two chimaeric molecules were also genetically engineered. In CD4-CD8, sequences corresponding to amino acids 1–177 of CD4 (product of exons 1–5) are fused to sequences encoding the hinge, transmembrane and cytoplasmic domains of human CD8 (exons 3–6); in CD4-LDL-R the CD4 ectodomain (amino acids 1–367) is fused to the transmembrane and cytoplasmic domains of the human low-density lipoprotein (LDL) receptor. These DNAs were inserted into the retroviral vector pMV7 (ref. 11) and were introduced into the A2.01 cell line, a CD4⁺ derivative of the human T cell leukaemic line, A3.01 (ref. 12), via a retroviral packaging system (see legend to Fig. 1). Individual clones expressing surface CD4 were identified by cytofluorometry following staining with fluorescein-conjugated monoclonal antibodies OKT4 or Leu3A.

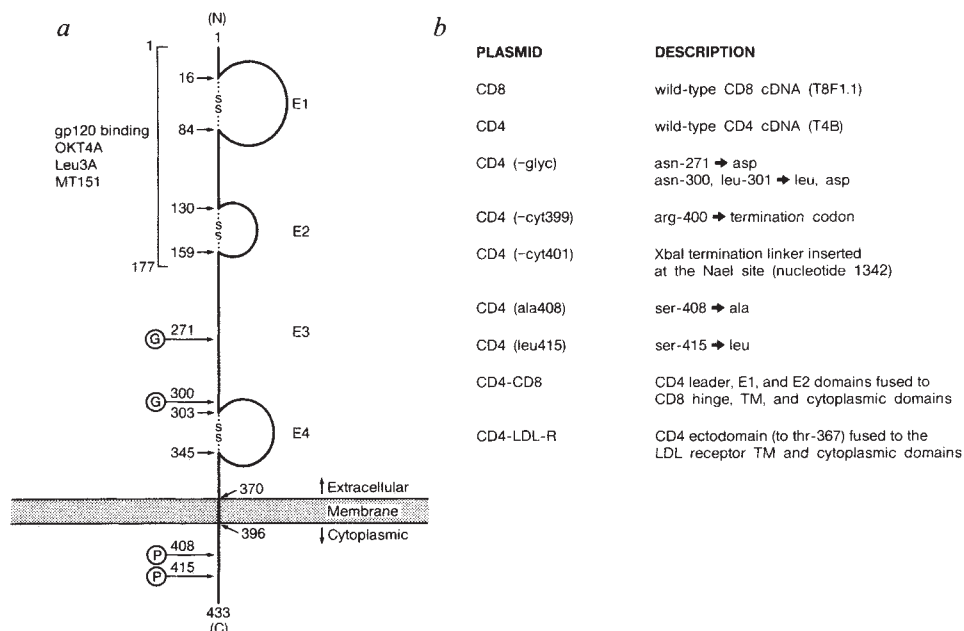
Activation of CD4⁺ cells with antigen or anti-receptor antibodies results in phosphorylation of one or more serine residues

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Fig. 1 *a*, Schematic diagram of the human CD4 glycoprotein. *b*, Description of the plasmid constructs. The wild-type DNAs were the 1.5 kilobase (kb) human CD8 cDNA (ref. 21) and the 1.7 kb *EcoRI-BamHI* fragment of the human CD4 cDNA (ref. 11). Numbering of the CD4 protein is based on the structure of the mature processed protein; this is a correction of the previously reported amino terminus¹¹, with lys-1 corresponding to asn-3.

Methods. Constructs were generated by site-directed mutagenesis²² as follows (numbering of nucleotides is according to the sequence of T4B (ref. 11)). CD4(-glyc): A → G at 961, C → T at 963 (introducing a *Bgl*III site); A → G at 1,048, T → A at 1,051, G → C at 1,053 (introducing an *EcoRV* site). CD4(-cyt399): T → C at 1,348, resulting in a termination codon. CD4(-cyt401): insertion of an *Xba*I termination linker (New England Biolabs) into the *Nae*I site at 1,342; this results in substitution of the cytoplasmic tail with four new amino acids, cys-397 being followed by the sequence pro-ser-leu-asp.

CD4(ala408): T → G at 1,372. CD4(leu415): AGT → CTC at 1,393–1,395 (creating a new *Xho*I site). CD4-CD8: a cDNA-genomic fusion of CD4 (the 0.6 kb 5' *EcoRI-Sac*I fragment of the CD4 cDNA fused to the 0.9 kb *Sac*I-*Bam*HI genomic fragment (containing exon 5 and the downstream intron)) was joined to a genomic fragment of CD8 (the 5.1-kb *Xho*I fragment containing exons 3–6). CD4-LDL-R: the 5' end of the CD4 cDNA was fused to the 3' end of the LDL-R cDNA at *Sal*I sites introduced at nucleotide 1,245 of CD4 (C → G at 1,248) and at nucleotide 2,349 of the human LDL receptor²³ (C → T at 2,350, C → G at 2,352, G → C at 2,354). Sequences of the mutant molecules were confirmed by the dideoxy chain termination procedure²⁴, cloned directionally into the *EcoRI-Hind*III sites of the retroviral vector pMV7 (formerly pMV6tkneo, ref. 11) and introduced into the ecotropic packaging cell line, Psi-2 (ref. 25), by calcium phosphate transfection. Supernatants of G418-resistant cells were used to infect an amphotropic packaging cell line, PA317 (ref. 26), in the presence of 8 µg ml⁻¹ polybrene. G418-resistant, CD4⁺ colonies of PA317 were treated with mitomycin c (10 µg ml⁻¹) for 2 h and were co-cultured overnight with A2.01 cells¹² (at a 1:1 cell ratio). A2.01 clones were selected for growth in 1.6 mg ml⁻¹ G418, tested for surface CD4 expression by cytofluorometry, and analysed by Southern blotting to confirm the identity of the introduced CD4 DNA (data not shown). Cells expressing the CD4-CD8 chimera bind to the anti-CD4 monoclonal antibody Leu3A and to gp120 (N. Landau, M. Warton and D.R.L., unpublished results), but not to OKT4 (which binds to the membrane proximal region of human CD4, ref. 27).



within CD4 and, subsequently, in internalization of the CD4 glycoprotein^{13,14}. Phorbol esters induce a similar response^{13,15}, suggesting that this is mediated by activation of protein kinase C. We analysed the ability of the mutant CD4 molecules to be internalized upon treatment with phorbol-12, 13-dibutyrate (Fig. 2, Table 1). A2.01 cells expressing wild-type CD4 exhibit almost complete loss of surface CD4, but no change in the level of class I MHC molecules, after treatment with phorbol ester for 4 h (Fig. 2, left panels). In contrast, CD4 is not internalized if its cytoplasmic domain is removed, as in the mutant CD4(-cyt401) (Fig. 2, middle panels). Replacement of the CD4 transmembrane and cytoplasmic domains with those of CD8 also abrogates internalization (CD4-CD8, Fig. 2, right panels). This result is consistent with the finding that phorbol ester-induced phosphorylation of CD8 is not accompanied by internalization of the CD8 glycoprotein¹⁴. Replacement of the serine residues at positions 408 or 415 results in a decreased, but significant, rate of internalization (data not shown), suggesting that more than one serine residue can be phosphorylated by protein kinase C. These results indicate that the cytoplasmic tail of CD4 contains sequence information necessary for phorbol-ester-mediated down-regulation of the CD4 protein.

To analyse the ability of mutant CD4 molecules to serve as HIV receptors, each cell line was exposed to HIV and monitored for subsequent infection by testing for the appearance of the p24^{gag} product of HIV. As shown in Fig. 3, neither untransfected A2.01 cells nor A2.01 cells expressing transfected CD8 glycoprotein were infected, whereas CD4⁺ A3.01 cells and A2.01 cells expressing wild-type or any of the mutant forms of CD4 were infected. Deletion of the cytoplasmic domain of CD4 (-cyt399 and -cyt401) did not prevent infection of the cells nor did removal of both of the *N*-linked glycosylation sites, substitu-

tion of cytoplasmic serine residues nor replacement of the transmembrane and cytoplasmic domains with those from the LDL receptor. Perhaps most surprising was the finding that the CD4-CD8 chimera, which contains only the amino-terminal 177 residues of CD4, is an effective receptor. These results confirm that the binding site for HIV is in the first two domains (E1 and E2) of CD4; and indicate that the remainder of the CD4 molecule is not required for HIV entry. They do not, however,

Table 1 Effect of CD4 mutations on HIV infectivity and CD4 internalization

Cells	Phenotype CD4	CD8	p24 ^{gag}	CD4 modulation with phorbols
A2.01	—	—	—	ND
A3.01	+	—	+	++
A2.01/CD8	—	+	—	—
A2.01/CD4	+	—	+	++
A2.01/CD4(-glyc)	+	—	+	ND
A2.01/CD4(-cyt399)	+	—	+	—
A2.01/CD4(-cyt401)	+	—	+	—
A2.01/CD4(ala408)	+	—	+	+
A2.01/CD4(leu415)	+	—	+	+
A2.01/CD4-CD8	+	+	+	—
A2.01/CD4-LDL-R	+	—	+	ND

A2.01 cells were infected with retroviral vectors containing the constructs described in Fig. 1. Surface phenotype was determined by cytofluorometry. Effect of phorbol esters and measurement of p24^{gag} levels are as described in Figs 2 and 3. ND: not determined.

* E1 and E2 domains from CD4; hinge, transmembrane, and cytoplasmic domains from CD8.

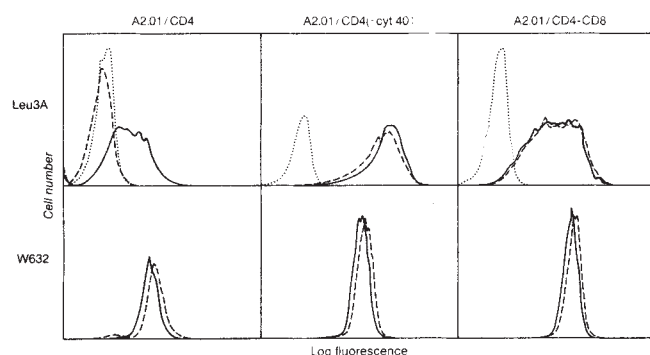


Fig. 2 Effect of phorbol ester on internalization of wild-type and mutant CD4 molecules. A2.01 cells expressing CD4, CD4(-cyt401) or CD4-CD8 were treated with phorbol-12,13-dibutyrate (PDB) and tested for expression of CD4 (using PE-Leu3A) and HLA (using F1-W632) at 4 h. Solid lines, untreated cells; dashed lines, PDB-treated cells; dotted lines, control (CD4⁻) A2.01 cells.

Methods. Cells were incubated at 37 °C in media with or without 1 μ M PDB (LC Services) 1 day after seeding at $2-5 \times 10^5$ cells ml^{-1} in Dulbecco's modified Eagle's medium with 10% fetal calf serum. After 4 h, 10^6 cells were transferred into PBS/1% BSA/0.1% sodium azide on ice, spun and resuspended in 100 μ l PBS/BSA/azide containing 0.5 μ l W632-FITC (Pel Freez) and 5 μ l Leu3A-PE (Becton-Dickinson). After 20 min on ice, the cells were washed, resuspended in 0.5 ml PBS/azide, and analysed on a Becton-Dickinson FACSAN.

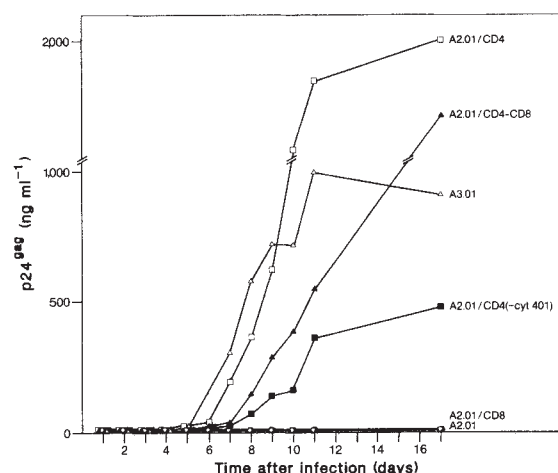


Fig. 3 Effect of CD4 mutations on infectivity with HIV.

Methods. Each cell line was incubated with 0.2 ml of HIV viral stock (LAV-BRU strain²⁸) per 10^6 cells. Following incubation with virus for 18 h, the cells were pelleted and treated twice with 1 ml of 0.25% trypsin/0.5 mM EDTA for 6 min at 37 °C. The cells were diluted to 10 ml in culture medium containing 10% fetal calf serum, washed three times, counted and resuspended at 1×10^4 cells per ml in culture medium (with 800 μ g ml^{-1} G418, if applicable). Duplicate 1 ml samples of each were immediately lysed by adding 50 μ l of a 20% solution of Triton X-100. This sample is the day 1 assay point. The contents of duplicate wells (cells and supernatants) from each cell line were harvested on the days shown. Infection was monitored by assaying the p24^{agg} antigen levels in an enzyme-linked immunosorbent assay²⁹.

rule out a role for these CD4 domains in regulating the rate of viral entry and viral production. The time course and extent of virus production in cells expressing mutant CD4 molecules varied among different clones (Fig. 3). These differences have not been assessed quantitatively due to variation in growth rates and in the stability of CD4 expression in these cells.

Our experiments demonstrate that the mutant CD4 molecules can function as effective HIV receptors even though they are not internalized in response to phorbol esters. These findings suggest that receptor-mediated endocytosis is not required for infection with HIV but do not exclude the possibility that the truncated CD4 molecules are still internalized in response to binding of virus. Other receptors, such as the epidermal growth factor and transferrin receptors, continue to function in ligand-induced endocytosis subsequent to mutation of cytoplasmic serines or threonines, whose phosphorylation is required for phorbol-ester-induced down-regulation^{16,17}. Removal of the cytoplasmic tail of the LDL, EGF and transferrin receptors, however, completely abrogates their internalization¹⁷⁻¹⁹. It is not yet known whether CD4, like these receptors, is selectively concentrated in coated pits. If CD4 internalization also proceeds via receptor-mediated endocytosis, truncation of its cytoplasmic domain would be expected to result in loss of HIV-induced, as well as phorbol-ester-induced, internalization. It is, however, possible that ligand-induced (or HIV-induced) internalization can proceed independent of the cytoplasmic domain through an association between CD4 and another plasma membrane protein. If HIV entry is mediated through such a complex, interaction of a second protein with CD4 would involve solely the E1 and E2 regions of CD4.

Speculation that HIV infection proceeds via receptor-mediated endocytosis has been supported by the finding that mouse and human cells differ in their ability to be infected. Whereas human cell lines transfected with human CD4 can be infected with HIV, murine cells expressing human CD4 can bind HIV but cannot be infected⁸. The block was shown to occur after binding of virus but prior to uncoating of the viral genome, as murine cells do not produce vesicular stomatitis viral (VSV) plaques upon infection with VSV(HIV) pseudotypes, but produce plaques subsequent to infection with VSV(MLV)

pseudotypes. Based on this result, it has been suggested that the human CD4 molecule interacts with a species-specific cytoplasmic element to facilitate entry of HIV (ref. 8). Our experiments suggest that, if a second molecule is required for viral entry, it does not interact with the cytoplasmic, transmembrane, or membrane-proximal external regions of CD4. Such a molecule could interact with either gp41 or gp120 subsequent to virus binding to the CD4 receptor and could, potentially, facilitate fusion of viral and plasma membranes. Alternatively, a second component may be required for uncoating of the viral genome subsequent to fusion and may be present in the cytoplasm of human cells. It is also possible that a dominant murine product inhibits a critical step in viral entry. Experiments with mouse-human somatic cell hybrids, designed to determine whether a second cellular component is necessary for HIV infection, are in progress.

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HIV-1 *tat* trans-activation requires the loop sequence within *tar*

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Human immunodeficiency virus (HIV-1) is the primary retroviral agent responsible for AIDS and related disorders worldwide. One of its identified gene products, *tat* protein, stimulates in *trans* the expression of all HIV-1 genes by several orders of magnitude¹⁻⁷. Cells infected with HIV-1 require *tat* protein to produce virus, suggesting that *trans*-activation is crucial for viral replication^{8,9}. The essential *cis*-acting site for *trans*-activation, termed *tar*, resides within the R region of the HIV-1 long terminal repeat (LTR), between -17 and +54 with respect to the initiation site of viral transcription^{10,11}. It is striking that the RNA encoded between +1 and +59 has the potential to form an extensive stem-loop secondary structure which, as a portion of the untranslated leader RNA, would be common to all HIV-1 mRNAs^{11,12}. We now present the results of nucleotide substitution experiments which suggest that *tat* *trans*-activation requires presentation of the sequence ⁺³⁰CUGGG⁺³⁴ in *tar* within the loop of a RNA hairpin structure.

Our efforts to identify the features of *tar* that are important for *tar*-dependent *trans*-activation in HIV-1 were stimulated by the report of *trans*-activation in HIV-2, a retrovirus closely related to HIV-1 which is responsible for a clinically indistinguishable immunodeficiency syndrome in West Africa¹³. Particularly intriguing has been the demonstration that *tat* protein from HIV-1 is capable of *trans*-activating constructs containing the *tar* region of HIV-2 (refs 13, 14). This heterologous activity inspired a search of the *cis*-acting regions of HIV-1 and HIV-2 for common features. Although direct comparison failed to uncover any regions of sustained sequence homology, further consideration did reveal that the RNA species encoded by HIV-2 *tar* could assume extensive secondary structure. Figure 1 shows a juxtaposition of the RNA structures proposed for the *tar* regions of HIV-1 and HIV-2. The single loop in the putative HIV-1 stem-loop structure and all three loops in one structure potentially adopted by the HIV-2 *tar* sequence are comprised of five to seven nucleotides which include the sequence CUGGG. The four stem moieties share no consistent features of either sequence or length. These observations suggested our initial working hypothesis that *tat* protein may mediate *trans*-activation via a *cis*-acting site which presents a CUGGG pentanucleotide in the loop context of an RNA stem-loop structure.

To test this hypothesis directly, we systematically altered each of the six nucleotides comprising the putative loop from HIV-1 and tested the resultant constructs for ability to support *tat*-

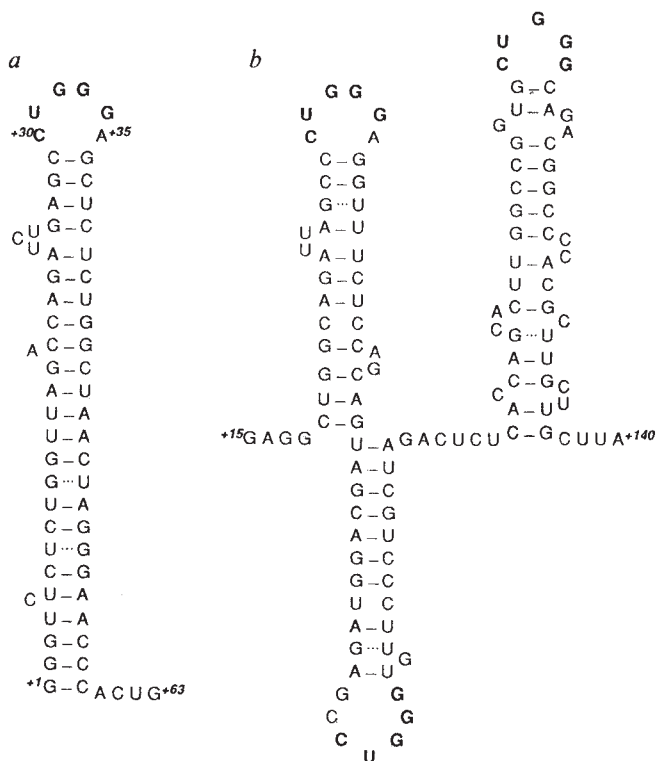


Fig. 1 Potential RNA secondary structures for the *tar* regions of HIV-1 and HIV-2. *a*, The *tar* region of HIV-1 has been drawn in its most stable secondary structure^{11,12}. *b*, The *tar* region of HIV-2 is capable of forming several secondary structures, one of which is shown. Similarities in loop sequences to HIV-1 *tar* are indicated in bold. The numbers refer to nucleotide positions with respect to the start of transcription.

dependent *trans*-activation. The assay we have employed measures CAT (chloramphenicol acetyl transferase) activity levels which increase dramatically when the gene, under the control of HIV-1 promoter and wild type *tar* sequences, is expressed in the presence of *tat* protein. A set of six *tar*-CAT expression plasmids (pHIVCATx) each of which differs from the wild-type *tar* region by a single nucleotide, and a *tat* expression plasmid (pcDEBtat) were constructed (Fig. 2). All changes in the HIV-1 *tar* region were introduced into the DNA by standard techniques as detailed in Fig. 2. Therefore, from this point forward, we shall refer explicitly to the relevant DNA sequence with intended implicit reference to the corresponding RNA species unless otherwise specified.

Figure 3 displays the stimulation of CAT gene expression achieved by HIV-1 *tat* protein in conjunction with either wild-type or mutant HIV-1 *tar* sequences; Table 1 provides a quantitative summary of the primary experimental data. The first set of mutant *tar* constructs explore the six nucleotides comprising the loop moiety of the proposed RNA stem-loop structure. The collection of corresponding functional assays is presented as Fig. 3a. It is apparent that pHIVCAT0, the wild type *tar* construct, supports substantial *trans*-activation. When compared to an extract of cells transfected with pHIVCAT0 alone, acetylation of chloramphenicol is 30-100-fold higher with an extract of cells co-transfected with pcDEBtat. In contrast, considerably less *trans*-activation is observed with pHIVCAT1 through pHIVCAT5, five constructs which each bear a single base substitution at one of five consecutive positions within the loop. These results indicate that the mechanism of *tat*-dependent *trans*-activation is exquisitely sensitive to the presence of the pentanucleotide CTGGG at positions +30 to +34. As pHIVCAT6, a construct

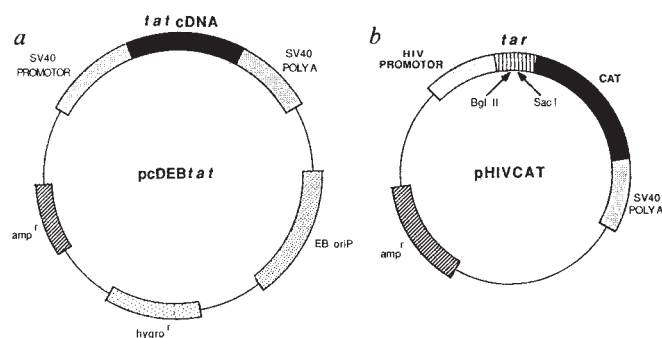


Fig. 2 Plasmids used to define features of HIV-1 *tar* essential for *trans*-activation by HIV-1 *tat* gene product. *a*, The *tat* expression vector, pcDEB*tat*. The coding region of a *tat* cDNA was excised as a *Sac*I fragment from the plasmid pCV-1 (gift of F. Wong-Staal) and inserted into the expression vector pcDEB (gift of H. Hayakawa). In the resultant construct, *tat* expression is regulated by the SV40 early promoter and SV40 RNA processing signals. Other noteworthy features of pcDEB*tat* including origins of replication and genes encoding selectable markers are as indicated. *b*, The collection of *tar*-CAT constructs, pHIVCAT_x, *x* = 0–9 and 11. A parent plasmid was constructed in pGEM by placing a reporter gene, chloramphenicol acetyl transferase (CAT), under the control of HIV-1 LTR sequences (U3 in entirety and R through +78, taken from pRIP 7, gift of M. McCune) which contain enhancer, promoter, and *tar* elements. SV40 sequences were included to provide essential RNA processing signals. The result, pHIVCAT, contained two unique restriction sites at positions which flank sequences encoding the proposed RNA loop: the *Bgl*II site is located at +19; the *Sac*I site is located at +34; and, the CUGGG pentanucleotide extends from +30 to +34, inclusive. Exploiting the fortuitous availability of restriction sites, we have used double-stranded, degenerate oligonucleotides to introduce nucleotide substitutions within the region of interest and thereby generate a collection of *tar*-CAT constructs, pHIVCAT0 through pHIVCAT9 and pHIVCAT11. In brief, complementary single-strand oligonucleotides were first annealed and then ligated to pHIVCAT linearized by digestion with *Bgl*II and *Sac*I. In some cases (pHIVCAT 6, 8, 9 and 11), pHIVCAT was digested with *Sac*I alone and the resultant 3' overhang was eliminated before digestion with *Bgl*II. The ligation mixture was used to transform *Escherichia coli* (strain MC1061); transformants were isolated by their resistance to ampicillin. The various *tar* mutants were identified by isolation and partial Maxam-Gilbert sequencing of the plasmid harboured by the individual transformants. After large-scale preparation of the selected mutants, the nucleotide sequence in the *tar* region of each was reconfirmed by Maxam-Gilbert sequencing.

Table 1 Quantitative levels of *trans*-activation observed with various *tar*-CAT constructs

<i>tar</i> -CAT construct	Sequence	<i>trans</i> -activation
pHIVCAT0	+20 AGATCTGAGCCTGGGAGCTCT+40	1.0
pHIVCAT1	*****A*****	<0.05
pHIVCAT2	*****A*****	<0.05
pHIVCAT3	*****T*****	<0.05
pHIVCAT4	*****T*****	<0.05
pHIVCAT5	*****T*****	<0.05
pHIVCAT6	*****C*****	0.7–1.5
pHIVCAT7	*****G*****	<0.05
pHIVCAT8	*****G*****C****	0.7–1.5
pHIVCAT9	*****TCG*****CGA**	0.3–0.4
pHIVCAT11	*****x*****C****	<0.05

Each *tar*-CAT construct tested for *trans*-activation competence is identified by name and by its DNA sequence in the region where alterations have been introduced. Nucleotides which differ from the wild-type sequence are specified by letter; nucleotides which correspond to the wild-type sequence are indicated by asterisks. The *trans*-activation competence of each construct is normalized with respect to the *trans*-activation activity observed for the wild-type plasmid pHIVCAT0, here defined as 1.0; the numbers reflect a range of typical values derived from four or more sets of parallel experiments. Bands were excised from TLC plates and counted in scintillation fluid to calculate the absolute values of chloramphenicol conversion efficiency which then yield absolute and normalized values of *trans*-activation. Variability can be attributed, at least partially, to the extreme disparity among the conversion efficiencies of the samples tested since the linear range of the CAT assay does not accommodate their simultaneous and accurate measurement.

positions +29 and +36. One of the *tar*-CAT expression vectors, pHIVCAT7, contains a single nucleotide substitution of G for C at +29; a second vector, pHIVCAT11, contains a single substitution of C for G at +36, and a third construct, pHIVCAT8, contains both of the substitutions at +29 and +36. The CAT expression assays with these constructs indicate that *tat*-mediated *trans*-activation is reduced by the elimination of base-pairing at this position, but reaches normal levels when the base-pairing is restored. This result argues for the necessity of a complementary interaction between the two nucleotides specified above, rather than their precise identity, to achieve wild-type levels of *trans*-activation.

Finally, we have extended the preceding analysis of regions adjacent to the putative loop sequence from a consideration of structural requirements to a consideration of sequence requirements. In plasmid pHIVCAT9, nucleotides occupying six positions, +27 through +29 and +36 through +38, have been coordinately changed to their Watson-Crick complements. Figure 3c shows that it supports *trans*-activation, but less efficiently than the wild type *tar* plasmid. Thus, the *tat*-dependent mechanism clearly lacks stringent sequence requirements in the six positions investigated. Rather, the consistent difference between the *trans*-activation capabilities of pHIVCAT9 (six base substitutions) and pHIVCAT8 (two base substitutions) suggests that the four additional changes may have infringed upon modulatory but non-essential determinants of *tat* function.

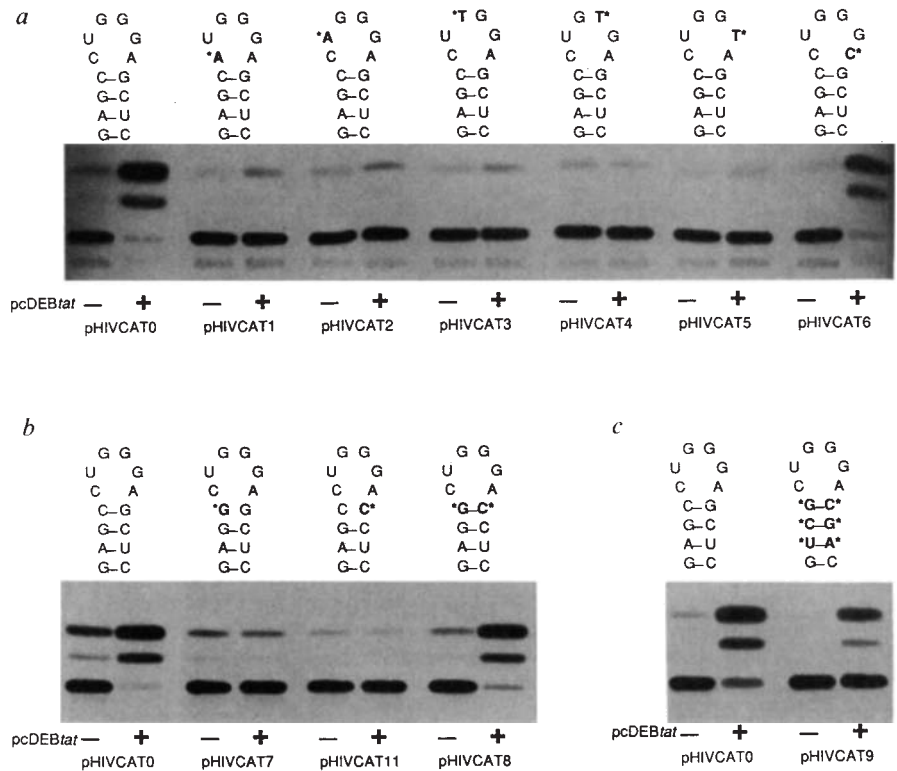
Note that there is no significant difference in the level of CAT activity between the wild type and any mutant *tar*-CAT construct in the absence of *tat*. Thus, the region extending from +27 to +38 appears to influence gene expression only through the *tat*-dependent *trans*-activation mechanism.

In conclusion, data derived from both sequence comparison and functional experiments have localized and defined features in the HIV-1 *tar* region that influence the ability of *tat* to activate gene expression in *trans*. Our results are entirely consistent with

containing a base alteration at position +35, demonstrates wild-type levels of *trans*-activation, the identity of the sixth and last position of the putative loop appears not to be so critical. The above results, derived from genetic experiments, clearly indicate that efficient *tat* function requires some critical sequence determinants in the region between +30 and +34, inclusive. Furthermore, they correlate well with observations derived from sequence comparisons between HIV-1 and HIV-2: whereas the essential CTGGG nucleotides are present in all three possible loop elements within the HIV-2 *tar* region, the non-essential A nucleotide is present only once (Fig. 1). We next consider whether this pentanucleotide element must be associated with a specific structural context for *tat* protein to stimulate gene expression in *trans*.

We have altered, first singly and then coordinately, the two bases immediately adjacent to the putative loop element. Only disruption of the base pair which initiates the stem is certain to perturb the proposed RNA secondary structure; nucleotide substitutions that disrupt base pairs internal to the stem would have indefinite and undeterminable effects. Figure 3b shows that Watson-Crick complements are indeed necessary in the pivotal

Fig. 3 CAT assays reflecting the ability of constructs bearing wild-type and mutant *tar* sequences to support *tat*-dependent trans-activation. Aliquots of 20 µg of pHIV-CATx (x = 0–9 and 11) were electroporated into 10⁷ Jurkat cells (human T lymphocytes) either without (–) or with (+) 20 µg of pcDEBtat. Cells were harvested and extracts prepared 48 h after transfection. The particular *tar*-CAT construct relevant to each pair of CAT assays is identified by name and illustrated by a portion of the corresponding RNA species in the region of nucleotide substitutions: altered bases are doubly highlighted by asterisk and bold-face type. Quantification of the observed trans-activation activity is presented in Table 1. Nucleotide substitutions have been introduced in *tar* to investigate (a) sequence requirements for the loop moiety of the proposed RNA stem-loop structure; (b) structural requirements for positions immediately flanking the proposed loop element; and (c) sequence requirements for regions adjacent to the proposed loop element.



the pre-existing evidence concerning the location and extent of the *cis*-acting site, in particular, the deletion analyses which established the minimal size of *tar* needed to support efficient levels of trans-activation. In the presence of an intact HIV-1 promoter, 3' deletions extending to +54 retain full competence for trans-activation whereas a deletion extending to +38 abolishes activity^{4,10,11}. These deletion studies therefore show that the preservation of *tar* function may be dependent upon the preservation of *tar* structure, particularly the potential for the encoded RNA to form an extensive stem-loop structure. Similar studies designed to explore the corresponding *cis*-acting site in HIV-2 have shown that 3' deletions, which gradually eliminate the sequences needed to produce secondary structure result in a progressive reduction of trans-activation efficacy¹⁵.

Although our findings, derived from either genetic analysis or sequence comparison, cannot unambiguously establish structure-function relationships, the internal consistency of the pooled observations argues in favour of the structural parameters of *tar* which we propose as essential for *tat* function. If the structural elements operated at the DNA level, one would necessarily postulate that the *tat* mechanism regulates gene expression at the transcriptional stage, possibly through a cruciform structure. The lack of precedence for the biological relevance of such a DNA structure, however, favours the involvement of a hairpin at the RNA level. Multiple investigators have heretofore reported that co-expression of *tat* results in elevated levels of *tar*-containing mRNAs, although there is substantial discrepancy as to whether the rise is commensurate with that observed for the protein product^{7,11,16–19}. Many models which one could postulate to account for the suggested increase in RNA levels necessitate an interaction between the regulatory machinery and the RNA species itself at either a co- or a post-transcriptional stage. In the HIV-1 system, the *tat* trans-activation mechanism could operate through the RNA form of *tar* to increase message production or message stability by facilitating elongation, preventing premature termination¹⁸, obstructing nucleases or loading ribosomes. Moreover, any portion of the *tat* trans-activation effect attributable to enhanced productivity of *tar*-containing mRNAs would similarly require

that *tar* functions in its RNA form. Our data do not discriminate among these various possibilities but simply define critical sequence and structural features within *tar* which probably operate at the RNA level.

It has generally been assumed that *tar* acts as a binding site for a protein, perhaps the *tat* gene product. But no protein-nucleic acid interaction between *tat* and *tar* has yet been demonstrated. Our collection of highly specific *tar* mutants should facilitate the investigation of precise mechanistic questions. Moreover, its characteristic activity profile may serve to identify related mechanisms of trans-activation involving *tar*.

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A region of SV40 large T antigen can substitute for a transforming domain of the adenovirus E1A products

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SV40 large T antigen contains a small region of amino acid sequence, conserved among the papovaviruses, that shows considerable similarity to conserved domain 2 of the adenovirus E1A oncogene, a domain which plays an important role in the E1A transforming functions. To learn whether the analogous SV40 T antigen sequences could substitute functionally for E1A domain 2, a chimaeric gene was constructed, coding for T antigen amino acid residues 101 to 118 in place of E1A domain 2. The resulting product showed much of the activity of the wild-type E1A products. It induced proliferation of primary BRK cells and cooperated with the *ras* oncogene to transform these cells fully. In addition, the chimaeric protein coprecipitated two cellular proteins whose specific binding to the E1A products depends on the presence of domain 2. The activity of the chimaeric product suggests that a similar functional unit exists in the transforming proteins of both SV40 and adenovirus, and that these proteins may exert their cell growth regulating effects through similar mechanisms.

The adenovirus early region 1A (E1A) gene plays an important role in adenovirus-mediated oncogenesis, and by itself is able to induce proliferation of quiescent primary rodent cells^{1,2}. The E1A gene has proved to be a particularly interesting model for studying structure-function relationships in a eukaryotic cell cycle control protein because its polypeptide products appear to contain readily distinguishable functional domains that have been highly conserved in the evolution of various adenovirus serotypes³.

The positions of the three conserved amino acid domains⁴ in the E1A products is illustrated schematically in Fig. 1. Domain 3 distinguishes the larger of the two major (13S and 12S) E1A cDNA products, and is dispensable for the function required to induce proliferation or transformation of primary baby rat kidney (BRK) cells⁵⁻⁸. Most of the nonconserved sequences are also dispensable for these functions, while sequences in domains 1 and 2 appear to be essential⁹⁻¹⁸. Several lines of evidence suggest that domains 1 and 2 are independent units. Domain 1 (like domain 3) can be removed selectively from the E1A products by differential splicing^{15,17}. Domains 1 and 2 can function independently of the spacing between them^{12,14,18}, and can cooperate even when expressed in separate polypeptides¹³.

Analysis of the structural requirements for domain 2 function has been facilitated by its small size (less than 20 amino acids) and its well-defined boundaries. To explore the possibility that this domain represents a functional unit used repeatedly in evolution, we considered whether a region of homologous sequence from a protein with similar biological activity could substitute for domain 2 functionally. For comparison, we chose the major tumour antigen (large T antigen) of SV40 virus, a member of the papovavirus family of DNA tumour viruses, which do not show a direct evolutionary relationship to the adenoviruses, but which have similar oncogenic properties^{19,20}.

Stabel *et al.*¹⁹ noted that the papovavirus large T antigens contain a sequence similar to E1A domain 2. The C-terminal half of domain 2 consists of a serine followed by a string of acidic residues. This motif, which occurs in several nuclear oncogene products^{21,22} constitutes a recognition site for a cellular protein kinase, casein kinase II (CK-II)²³, which may play a role in cell cycle control (ref. 24 and D. Carroll, E. Moran, N. Santoro and D. Marshak, in preparation). The N-terminal portion of E1A domain 2 contains the adenovirus-conserved

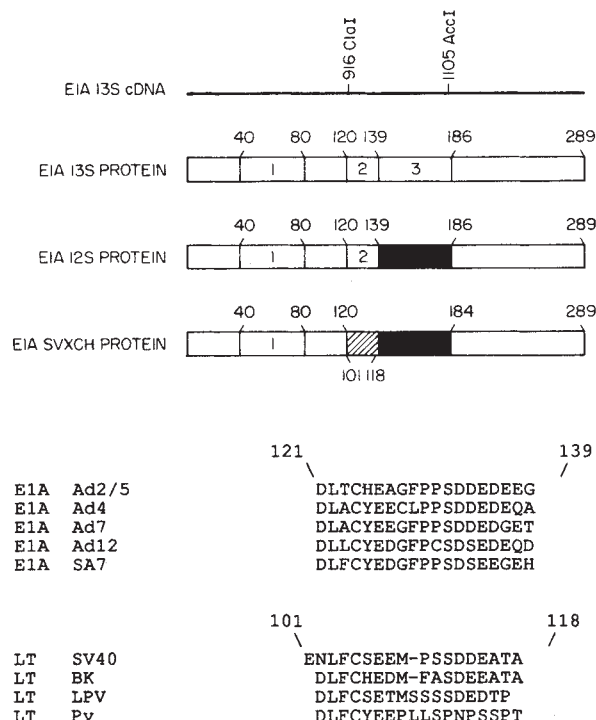


Fig. 1 Schematic representation of wild-type and mutant products of the E1A region and alignment of sequences from E1A proteins and large T antigens. The nucleotide sequence of the coding region of the 13S wild-type E1A cDNA, which encodes a 289 amino acid product, is represented by the top line. Nucleotide positions of selected restriction sites are indicated above the line. The protein products encoded by several E1A variants are indicated by the open bars, with the positions of the conserved domains (1, 2 and 3) indicated within them. A unique *ClaI* site occurs at the start of domain 2, and a unique *AccI* site occurs near the end of domain 3. Selected amino acid position numbers within the E1A variants discussed in the text are indicated above the bars; position numbers refer to the position as it occurred in the 289 amino acid product. The direction of the marks indicates to which side of the boundary the designated amino acid position lies. The 243-amino-acid 12S wild-type product is represented as an in-frame deletion of 46 amino acids from the 13S product. Deleted sequences are represented by blackened regions. To construct the chimaera, the *ClaI* to *AccI* fragment was removed from a 13S E1A cDNA construct, and replaced with complementary synthetic oligonucleotides encoding amino acid residues 101 through 118 of SV40 large T antigen. The resulting product, pE1A.SVXCH, codes for residues 101 through 118 of T antigen inserted between amino acid positions 120 and 184 of the E1A 13S cDNA product. As this construction deletes almost all of the 13S unique region, the chimaeric protein more closely resembles the 12S than the 13S E1A product. The position of the SV40 large T antigen sequences is indicated by the hatched region. Amino acid position numbers, indicated below the hatched region, refer to the positions as they would occur in T antigen. The lower part of the figure shows the domain 2 sequences from human adenovirus serotypes 2/5, 4, 7, 12 and simian adenovirus SA7 (refs 4, 31) aligned with homologous sequences from SV40, BK, lymphotropic papovavirus (LPV) and mouse polyomavirus (Py) (refs 32, 33). The alignments shown are adapted from data assembled by Stabel *et al.*¹⁹. In the present figure single amino acid gaps have been introduced to maximize the homology. The position numbers of the first and last amino acids of the Ad2/5 and SV40 sequences are indicated above the amino acid symbols.

sequence DLXCXE. Both of these elements occur in the papovaviruses, suggesting that the papovavirus T antigens may contain a structural analogue of E1A domain 2. (The relevant T antigen and E1A sequences are shown in Fig. 1.)

To determine whether this region of SV40 T antigen can fulfil the requirements for domain 2 function, domain 2 of E1A in plasmid and virus backgrounds was replaced with the analogous

Table 1 Immortalization functions of E1A variants

Virus	Mitotic index (%) (48 h)	Virus establishment* (3 weeks)	Plasmids	<i>ras</i> cooperation†
mock	0.09	0	<i>ras</i> + E1A.WT	27
12S.WT	7.0	22	<i>ras</i> + 12S.WT	20
E1A.SVXCH	4.5	2.3	<i>ras</i> + E1A.SVXCH	11
E1A.CXdl	<0.2	0	<i>ras</i> + E1A.928	0
12S.928	<0.2	0	<i>ras</i> + E1A.CXdl	0
			<i>ras</i> + pUC18	0

* Average number of colonies per plate.

† Average number of foci per 5 plates.

T antigen region as described in Fig. 1. The activities of the products of the resulting chimaeric gene, designated E1A.SVXCH, and the wild-type E1A products were compared in several assays in which E1A activity depends on the function of conserved domain 2. Two domain 2 mutants described previously^{12,25}, E1A.CXdl (a total deletion of domain 2) and pm928 (a single missense mutation, *cys* → *gly* at position 124), are shown for comparison.

The ability to induce proliferation of BRK cells was assessed quantitatively by determining the mitotic index. As shown in Table 1, infection with 12S wild-type (12S.WT) virus induces rapid proliferation, detectable by a sharp increase, ~70-fold, in the number of mitotic cells within 48 hours post-infection. Infection of these cells with the chimaeric virus, E1A.SVXCH, induced a similar sharp increase in the number of mitotic cells, at least 60% of the number seen after infection with 12S wild-type virus.

The ability to establish BRK cells was assayed by determining the number of cell colonies that continued to proliferate several weeks after infection (Table 1). The E1A.SVXCH virus was capable of establishing long-term growth, inducing about 10% of the number of colonies established by wild-type virus at the end of 3 weeks.

The E1A wild-type and chimaeric products were also compared for their abilities to cooperate with the *ras* oncogene to induce foci of morphologically transformed BRK cells in a plasmid cotransfection assay. The E1A.SVXCH plasmid functioned efficiently in this assay, showing 30–60% of wild-type activity (Table 1). The general morphology and growth characteristics of the colonies established by the E1A.SVXCH virus, or the transformed foci induced by the chimaeric plasmid in cooperation with the *ras* gene, were not noticeably different from the corresponding colonies induced by the 12S.WT constructs. The domain 2 mutants, E1A.CXdl and pm928, show virtually no activity in any of these assays.

The properties of the E1A.SVXCH virus were assessed further in other functions in which E1A domain 2 plays a role. Although domain 2 is not essential for the induction of DNA synthesis in BRK cells, the onset of DNA synthesis is delayed significantly during infection with the E1A.CXdl or 12S.928 viruses compared with 12S.WT infection²⁵. The E1A.SVXCH virus induced DNA synthesis in these cells with the same kinetics as 12S.WT (Fig. 2). In addition, BRK cells infected with the E1A.CXdl virus exhibit an unusual pattern of cell-cycle distribution when examined using a fluorescence-activated cell sorter¹³. The E1A.SVXCH variant, however, functioned indistinguishably from 12S.WT in this assay (data not shown).

Monoclonal antibodies specific for the E1A polypeptides precipitate, in addition to the E1A proteins, a series of cellular proteins from adenovirus-infected HeLa cells^{26,27}. Coprecipitation of two of the most prominent of these, those with estimated relative molecular masses (M_r) of ~105,000 and 107,000 (105K and 107K) depends on the presence of domain 2 sequences in the E1A polypeptides (Fig. 3, below, and P. Whyte, N. Williamson and E. Haslow, in preparation).

The ability of the chimaeric protein to bind to cellular proteins was assessed in infected HeLa cells. Ad5dl309 is the parental virus for all the E1A mutants discussed in this report, and contains a wild-type E1A gene. As shown in Fig. 3, immunoprecipitation of E1A products from Ad5dl309 (lane 3) or 13S.WT (lane 4) infected cells shows specific coprecipitating bands in the range of 92K to 116K. These bands are also present in immunoprecipitations from 12S.WT (lane 5) or E1A.NCdl (lane 6) infected cells. These variants contain deletions immediately upstream (E1A.NCdl deletes residues 86–120 (ref. 12)) or downstream (12S.WT deletes residues 140–185) of domain 2. These bands are absent or only poorly detectable upon immunoprecipitation of the E1A.CXdl (lanes 7 and 9) or 12S.928 (lane 8) products. The pattern of coprecipitating bands seen upon immunoprecipitation of the E1A.SVXCH chimaeric products (lane 10) is similar to that seen after immunoprecipitation of the wild-type E1A products. A small amount of nonspecific coprecipitating product was apparent in the control lane running very close to the slower running of the two specific products in the 92K to 116K range, and may contribute to the band observed coprecipitating in this range with the domain 2 mutants. No band corresponding to the faster running band seen with the wild-type immunoprecipitates could be detected in the domain 2 mutant lanes, even after longer exposures.

The ability of the chimaeric protein to bind cellular products whose association with the E1A products depends on the structure of domain 2 argues that the chimaeric protein can assume a tertiary structure similar to the wild-type E1A products. And the recent identification of the 105K cellular product as the retinoblastoma gene product²⁸ supports the suggestion that these

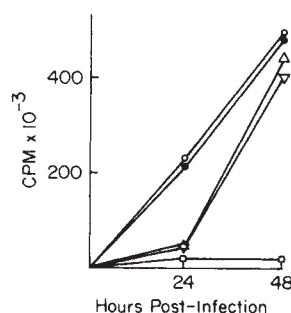


Fig. 2 DNA synthesis in infected primary BRK cells. Incorporation of ³H-thymidine into trichloroacetic acid precipitable counts was measured in primary BRK cells, or BRK cells mock infected (□), or infected with 12S.WT (●), E1A.SVXCH (○), E1A.CXdl (△), or 12S.928 (▽) virus, and labelled for 24-h periods ending at the time points indicated in the figure (see ref. 25 for methods). In this experiment and the virus experiments presented in Table 1, infections were done at a multiplicity of either 5 or 10 plaque-forming units (PFU) per cell, and the data reported are the averages of three independent experiments.

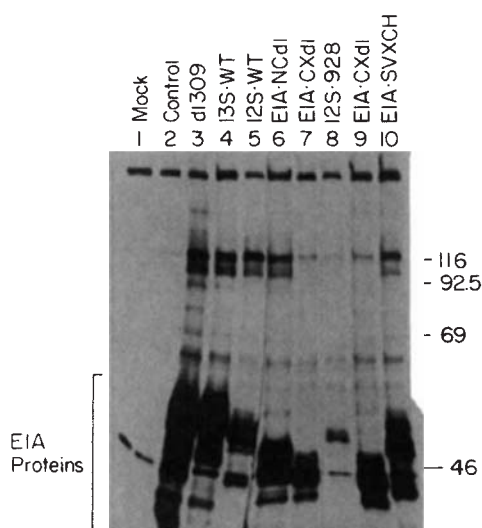


Fig. 3 Immunoprecipitation of E1A variant products in virus-infected HeLa cells. Protein extracts from HeLa cells mock-infected, or infected at a multiplicity of 100 PFU per cell with wild-type or mutant viruses as indicated in the figure, and described in the text, were immunoprecipitated with E1A fusion protein specific mouse monoclonal antibodies, series 73 as described²⁷ and separated on 10% SDS-polyacrylamide gels. Numbers on the right indicate the position of molecular weight markers (K). The region where the heterogeneous products of the E1A gene run is indicated on the left. The control lane contains a protein extract from Ad5dl309 infected cells, immunoprecipitated with a control mouse monoclonal antibody, Pab416, which does not recognize the E1A products.

interactions are significant for the cell cycle regulating functions of the E1A products.

The SV40 T antigen and Ad5 E1A regions compared here share less than 50% amino acid identity (Fig. 1), yet the chimaeric protein is biologically active. In contrast, a single amino acid substitution in one of the conserved residues (pm928:cys₁₂₄→gly) in Ad5 E1A is nearly as defective as a complete deletion of domain 2. The present demonstration that a functional substitution can be made between this region of T antigen and one of the transformation domains of the E1A gene products, suggests that similar protein structure and biochemical mechanisms underlie the transforming functions common to both proteins. Very strong support for this suggestion comes from the recent finding that T antigen binds the retinoblastoma gene product in CV-1 cells²⁹. Computer analyses also support the prediction that the E1A product and T antigen regions compared here have similar secondary structures²².

Further genetic evidence supporting the hypothesis that E1A domain 2 represents a functional motif used repeatedly, at least among the DNA tumour viruses, comes from the recent report that the deduced amino acid sequence of the product encoded by the human papillomavirus type 16 (HPV-16) E-7 gene, which encodes transformation functions similar to adenovirus E1A, contains a region which exhibits a striking degree of identity with the conserved residues in the transforming domains of the E1A products, particularly domain 2, and which includes the DLXCXE motif³⁰. Although the functional significance of this region in the HPV-16-E-7 gene product has not yet been determined, the convergence of these results makes it tempting to speculate that similar functional motifs and mechanisms underlie the activity of transforming genes from different classes of DNA tumour viruses which did not appear to be structurally related until the significance of specific small regions in the products of the E1A gene was appreciated.

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Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation

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A new Ca²⁺/calmodulin-dependent protein kinase has been recently discovered in mammalian cells^{1,2}. The major substrate of this kinase, a protein of relative molecular mass (*M_r*) ≈ 100,000 (100K), has been identified as elongation factor 2 (EF-2)^{3,4}, which participates in protein synthesis. The *in vivo* activity of the EF-2 kinase depends upon growth factors and other agents affecting the level of Ca²⁺ and cAMP^{5,6}. Its effect on EF-2 activity, however, remained obscure. This work shows that the phosphorylation of EF-2 by the EF-2 kinase results in a drastic inhibition of polyphenylalanine synthesis in poly(U)-directed translation. Phosphorylated EF-2 is completely inactive in translation and, moreover, inhibits the activity of non-phosphorylated EF-2. Dephosphorylation of EF-2 by phosphatase restores its activity. Hence, the phosphorylation of EF-2 directly affects the elongation stage of translation and thus represents a novel mechanism of translational control.

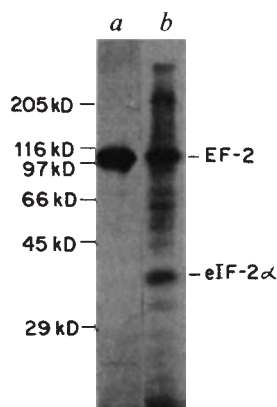


Fig. 1 Endogenously phosphorylated proteins in the ribosome-free extract (*a*) and crude cell-free translation system (*b*) from rabbit reticulocytes. A radioautograph of gels after SDS electrophoresis is shown.

Methods. The ribosome-free extract and crude lysate of rabbit reticulocytes were obtained as described^{3,8}. The phosphorylation reaction in *a* was carried out in 50 mM HEPES-KOH buffer, pH 7.6, containing 10 mM magnesium acetate, 5 mM DTT, 50 μ M [γ -³²P]ATP (1,500 c.p.m. μ mol⁻¹) and 4 μ l of the ribosome-free extract in a final volume of 40 μ l. The sample was incubated for 2 min at 30 °C. The reaction was stopped by the addition of 10 μ l of SDS-containing electrophoresis buffer and 20 μ l was loaded on the gel. The phosphorylation reaction in *b* was in 20 mM HEPES-KOH buffer, pH 7.6, containing 150 mM potassium acetate, 1.2 mM MgCl₂, 10 mM creatine phosphate, 1 mM [γ -³²P]ATP (1,000 c.p.m. μ mol⁻¹), 0.2 mM GTP, 0.5 mM spermidine, 1.0 mM DTT, a mixture of 20 amino acids (40 μ M each) and 22 μ l of reticulocyte lysate in a final volume of 30 μ l. The sample was incubated for 10 min at 37 °C. The reaction was stopped by the addition of SDS-containing electrophoresis buffer and 2.5 μ l was loaded on the gel. Electrophoresis was as described³. Dried gels were exposed on the Kodak film at -70 °C for 48 h.

Reversible phosphorylation of proteins is considered to be one of the main ways by which protein biosynthesis is regulated. Thus a study of phosphorylation of the translation initiation and elongation factors might provide insights into mechanisms of translational control. To date, eukaryotic initiation factor 2 (eIF-2) was the only protein participating in translation phosphorylation which had been demonstrated to affect its functional activity (see ref. 7 for recent review). In contrast, phosphorylation of the elongation factors with consequent changes in their activity has not been described.

We recently reported phosphorylation of EF-2 (ref. 3) in a rabbit reticulocyte ribosome-free extract, as well as in a crude lysate containing all the components sufficient for translation^{3,4,8} (see Fig. 1). The protein kinase responsible for EF-2 phosphorylation, called EF-2 kinase⁴, has been identified and partially purified from rabbit reticulocytes⁴. It has a M_r of $\approx$ 140K, as determined by gel filtration, and phosphorylates threonine residues in EF-2 (ref. 4). The EF-2 kinase is active only in the presence of Ca²⁺ and calmodulin, but it differs by a number of criteria from all known types of Ca²⁺/calmodulin-dependent protein kinases⁴. It does, however, have the same properties as Ca²⁺/calmodulin-dependent protein kinase III discovered in various mammalian tissues^{1,2}. EF-2 kinase shows strong substrate specificity for EF-2 as other known protein kinases are unable to phosphorylate EF-2 to a significant extent, and EF-2 kinase does not phosphorylate substrates other than EF-2 (ref. 2 and our unpublished observations).

Reticulocyte lysate has been shown to contain a significant proportion of phosphorylated EF-2, sometimes up to 50% of the total EF-2 (refs 4, 8). Endogenously phosphorylated EF-2 could be separated from non-phosphorylated EF-2 by ion exchange chromatography⁹, enabling the effect of EF-2 phos-

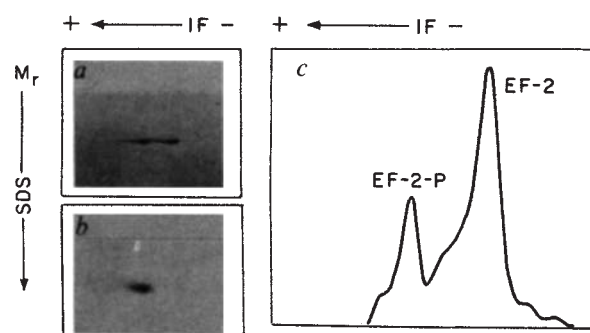


Fig. 2 Separation of phosphorylated and non-phosphorylated forms of EF-2 by isoelectrofocusing. *a*, Coomassie G-250-stained gel after two-dimensional separation¹² of the EF-2 preparation incubated with EF-2 kinase and 50 μ M [γ -³²P]ATP (500 c.p.m. μ mol⁻¹). *b*, Radioautograph of this gel (12-h exposure). *c*, Result of gel scanning after isoelectrofocusing. The gel column was stained with 0.05% Coomassie R-250, 0.1% CuSO₄ in a 3:1:6 (v/v/v) ethanol:acetic acid:water mixture for 3 h and the gels were washed in the ethanol:acetic acid:water solution for 4 h. The gel was scanned using a 2202 Ultrascan laser densitometer (LKB, Sweden). **Methods.** EF-2 (2 μ g) was incubated for 2 min at 30 °C with 1 μ g of partially purified EF-2 kinase and with [γ -³²P]ATP. The EF-2 kinase was prepared as follows. The rabbit reticulocyte ribosome-free extract was layered on a column with DEAE-cellulose (DE-52, Whatman) equilibrated with buffer A (10 mM Tris-HCl, pH 7.6, 1 mM MgCl₂, 50 mM KCl, 7 mM β -mercaptoethanol and 10% glycerol). EF-2 kinase was eluted by buffer A with 600 mM KCl. The eluate was diluted threefold with the same buffer and layered on a Mono Q column (Pharmacia) equilibrated with buffer A containing 200 mM KCl; EF-2 kinase was eluted by the KCl linear gradient (200–600 mM). Fractions with active EF-2 kinase, eluted at about 450 mM KCl, were frozen in liquid nitrogen and stored at -70 °C. The conditions of phosphorylation reaction were described previously^{3,4}.

phorylation on its activity to be studied. Testing different ratios of phosphorylated and non-phosphorylated EF-2 in poly(U)-directed polyphenylalanine synthesis has revealed that the phosphorylated EF-2 is inactive in translation⁹. These investigations lacked evidence that it was the Ca²⁺/calmodulin-dependent EF-2 kinase that phosphorylated EF-2, however, and so we have carried out a direct experiment using a partially purified preparation of the kinase.

EF-2 was purified from the ribosome-free extract of rabbit reticulocytes by successive chromatographies on RNA-Sepharose, hydroxyapatite and Mono Q (ref. 9). The EF-2 preparation was homogeneous according to electrophoresis with sodium dodecyl sulphate. Isoelectrofocusing, however, divided it into two bands corresponding to phosphorylated and non-phosphorylated EF-2 (see Fig. 2).

EF-2 kinase was also purified from the ribosome-free extract of rabbit reticulocytes (see legend to Fig. 2). The kinase activity was strictly dependent upon Ca²⁺ and calmodulin. Incubation of EF-2 with EF-2 kinase resulted in the phosphorylation of a portion of the factor as reflected in the increase of the band with a more acidic isoelectric point.

Figure 3 shows the kinetics of polyphenylalanine synthesis in the poly(U)-directed translation system on addition of EF-2 preparations containing different proportions of the phosphorylated form. The decrease of the non-phosphorylated EF-2 from 70% to 50% resulted in the threefold reduction of the rate of polyphenylalanine synthesis, and a preparation containing only 30% non-phosphorylated EF-2 was completely inactive. The inhibition of polyphenylalanine synthesis was not caused by any impurities in the kinase preparation, as kinase inactivated by the calmodulin antagonist, trifluoperazine (150 μ M) did not inhibit translation (in fact it was slightly stimulated).

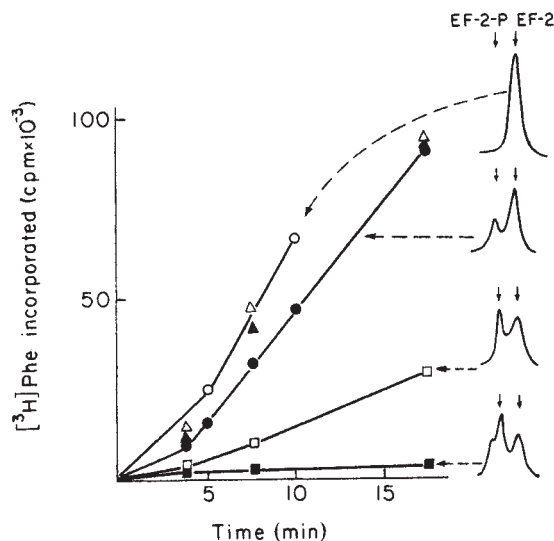


Fig. 3 Dependence of polyphenylalanine synthesis kinetics in the poly(U)-directed translation system on the addition of the EF-2 preparations with different proportions of the phosphorylated and non-phosphorylated forms of EF-2. (●) 30:70; (□) 50:50; (▲) 30:70; (Δ) 30:70; (■) 70:30; (○) 0:100; see below for details. The results of scanning the stained gels after isoelectrofocusing of the EF-2 preparations used in the given experiments are shown at the right.

Methods. In all experiments 0.4 μ g of EF-2 was used and other components were in excess. The incubation mixture (100 μ l) contained 3 pmol of ribosomes (an equimolar mixture of 40S and 60S subunits from rabbit reticulocytes), 10 μ g of poly(U) (Boehringer), 3 μ g of EF-1 from rabbit reticulocytes, 50 μ g of crude *Escherichia coli* transfer RNA aminoacylated with [3 H]phenylalanine (2,000 c.p.m. per 1 μ g of tRNA) in 20 mM Tris-HCl buffer, pH 7.6 with 100 mM KCl, 10 mM MgCl₂, 2 mM DTT and 1.2 mM GTP. Incubation was carried out at 37 °C and stopped by the addition of 5% trichloroacetic acid. The mixture was boiled for 10 min and filtered through GF/C glass filters (Whatman). The radioactivity of dry filters was determined by liquid-scintillation counting. Every point corresponds to the mean value obtained in two independent experiments. Separate treatments were as follows. (●) Original EF-2 preparation (8 μ g) incubated for 20 min at 30 °C in buffer B (50 mM HEPES KOH, 5 mM Tris-HCl pH 7.6, 10 mM MgAc, 60 mM KCl, 150 μ M CaCl₂, 0.5 mM ATP, 10 μ g ml⁻¹ bovine brain calmodulin, 6 mM DTT, 5% glycerol), final volume 180 μ l. (□) Original EF-2 preparation (8 μ g) was incubated with 3 μ g of EF-2 kinase for 20 min at 30 °C in buffer B, final volume 180 μ l. (▲) Original EF-2 preparation (8 μ g) was incubated with 3 μ g of EF-2 kinase as above, except that 150 μ M trifluoperazine was added. (Δ) Original EF-2 preparation (8 μ g) incubated in buffer B in 180 μ l with 150 μ M trifluoperazine for 20 min at 30 °C. (■) Original EF-2 preparation (8 μ g) incubated with 7 μ g of EF-2 kinase for 20 min at 30 °C in buffer B in 180 μ l. (○) Original EF-2 preparation (2 μ g) incubated for 25 min at 30 °C with 25 μ g of alkaline phosphatase from calf intestine (Boehringer, grade II) in buffer C (20 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 100 mM KCl, 2 mM dithiothreitol), final volume 88 μ l.

Calmodulin, Ca²⁺ and ATP at the concentrations used in this study have no influence on the rate of polyphenylalanine synthesis (data not shown). Incubation of the EF-2 preparation with alkaline phosphatase stimulated EF-2 activity, and was accompanied by the disappearance of the phosphorylated EF-2 form (see Fig. 3).

When EF-2 pre-incubated with EF-2 kinase was subsequently incubated with alkaline phosphatase, EF-2 activity was restored to that of completely dephosphorylated EF-2 (Table 1). Thus, the dephosphorylation of EF-2 restores its activity.

All these results demonstrate that EF-2, when phosphorylated by the EF-2 kinase, is inactive in translation. Moreover, Fig. 3 shows that polyphenylalanine synthesis is inhibited to a greater extent than the decrease in non-phosphorylated EF-2. It is thus

Table 1 Restoration of the activity of phosphorylated EF-2 after incubation with alkaline phosphatase

	[3 H]Phe incorporated c.p.m.	
	5 min	10 min
Original EF-2 preparation	11,646	34,677
EF-2 + EF-2 kinase	8,871	25,627
EF-2 + EF-2 kinase + alkaline phosphatase	26,666	64,495

The original EF-2 preparation used in this experiment contained 35% phosphorylated and 65% non-phosphorylated EF-2 forms. The preparation (2 μ g) was incubated with 2 μ g of EF-2 kinase at 30 °C for 4 min in buffer D (30 mM HEPES KOH, pH 7.6, 10 mM MgAc, 150 μ M CaCl₂, 0.5 mM ATP, 10 μ g ml⁻¹ bovine brain calmodulin, 5 mM dithiothreitol (DTT)) and for 10 min in buffer E (15 mM HEPES KOH, 20 mM Tris-HCl, pH 7.6, 50 μ M CaCl₂, 10 mM MgCl₂, 3 mM MgAc, 120 mM KCl, 4 mM DTT, 60 μ M trifluoperazine (the second row)). The EF-2 preparation preincubated with EF-2 kinase at 30 °C for 4 min in buffer D was incubated with 70 μ g of alkaline phosphatase in buffer E for 10 min at 30 °C (the third row). Original EF-2 preparation (2 μ g) was incubated in buffer D for 4 min and buffer E for 10 min (the first row). The final volume in all the incubations was 164 μ l. An aliquot (0.4 μ g) of EF-2 was added to the translation system in each experiment. The reaction was carried out as described in Fig. 3.

Table 2 Inhibition of the activity of non-phosphorylated EF-2 by phosphorylated EF-2

	[3 H]Phe incorporated c.p.m.	
	7 min	
Original EF-2 preparation (0.25 μ g)	14,794	
Original EF-2 preparation (0.25 μ g) + phosphorylated EF-2 (1 μ g)	10,596	
Original EF-2 preparation (0.25 μ g) + phosphorylated EF-2 (2 μ g)	8,634	

The original EF-2 preparation used in this experiment was completely dephosphorylated. This preparation was mixed with various amounts of fully phosphorylated EF-2. Phosphorylated EF-2 was obtained by incubating 50 μ g of EF-2 with 5 μ g of EF-2 kinase at 30 °C for 40 min in buffer D in a final volume of 250 μ l. The translation assay was carried out as described in Fig. 3.

probable that the phosphorylated EF-2 is not only inactive in translation, but also inhibits it.

To test this directly, we studied the effect of fully phosphorylated EF-2 on the activity of non-phosphorylated EF-2 and found that the addition of increasing amounts of phosphorylated EF-2 markedly suppressed EF-2 activity (Table 2). This phenomenon may have important physiological implications, as it is not necessary to phosphorylate EF-2 completely to shut off translation.

Why does EF-2 become inactive after phosphorylation? This question is not easy to answer as the mechanism of EF-2 function is intricate and poorly understood. Our working hypothesis is that phosphorylation of EF-2 alters its interaction with the ribosome and makes it unable to catalyse translocation. If this is true, the inhibition of non-phosphorylated EF-2 by its phosphorylated form could be due to the competition between these two EF-2 forms for a common binding site on the ribosome.

Reversible EF-2 phosphorylation is undoubtedly a physiologically relevant mechanism. As has been found earlier⁸, and as shown in Fig. 1, intensive phosphorylation of EF-2 is observed in a highly active non-fractionated translation system from rabbit reticulocytes. Moreover, the addition of 5 mM cAMP to such a system led to a three- to fivefold activation of endogenous protein synthesis, which correlates with the drastic dephosphorylation of EF-2 (ref. 8). This observation seems to reflect the *in vivo* situation as recently it has been shown^{6,10} that treatment of PC-12 cells with a nerve growth factor, or with

agents that increase the intracellular cAMP concentration, leads to dephosphorylation of EF-2.

Another example of an *in vivo* system where reversible EF-2 phosphorylation is implicated is the mitogenic stimulation of cells. A dramatic transient increase of EF-2 phosphorylation has been observed⁵ after mitogenic stimulation of fibroblasts, which ran parallel to but lagged slightly behind the intracellular Ca^{2+} transient.

Parallel studies of the elongation rate changes were not done in any of the above examples. Nevertheless, these results, together with those reported here, strongly indicate that the reversible EF-2 phosphorylation may serve as a link between intracellular second messengers such as cAMP and the rate of translation.

Finally we would like to note that, after this paper was submitted, a communication by Nairn and Palfrey¹¹ reported that EF-2 from rat pancreas, that has been phosphorylated by EF-2 kinase (Ca^{2+} /calmodulin-dependent protein kinase III) from the same source, is almost completely inactive in poly(U)-directed translation.

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Phosphatidylglycerol is involved in protein translocation across *Escherichia coli* inner membranes

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Newly synthesized proteins to be exported out of the cytoplasm of bacterial cells have to pass across the inner membrane. In Gram-negative bacteria ATP^{1,2}, a membrane potential^{3,4}, the products of the *sec* genes⁵ and leader peptidases^{6,7} (enzymes which cleave the N-terminal signal peptides of the precursor proteins) are required. The mechanism of translocation, however, remains elusive. Important additional roles for membrane lipids have been repeatedly suggested both on theoretical grounds^{8–11} and on the basis of experiments with model systems^{12–14} but no direct evidence had been obtained. We demonstrate here, using mutants of *Escherichia coli* defective in the synthesis of the major anionic membrane phospholipids, that phosphatidylglycerol is involved in the translocation of newly synthesized outer-membrane proteins across the inner membrane.

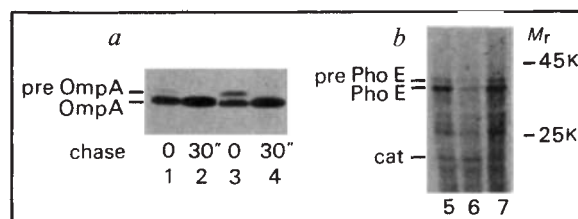


Fig. 1 Effect of PG and CL content on the *in vivo* synthesis and translocation of *E. coli* outer membrane proteins. *a*, Plasmid pHD102 containing cells of wild-type strain CE1224 (lanes 1 and 2, high PG and CL) and *pgsA3*-containing strain CE1250 (lanes 3 and 4, low PG and CL) were grown in a synthetic medium²⁷ at 43°C, until a low PG content was reached in CE1250. Cells were then pulse-labelled with [³⁵S]methionine at 37°C for 30 s (ref. 28), followed by a chase with unlabelled methionine for the indicated time period. After trichloroacetic acid precipitation the samples were immunoprecipitated using rabbit antiserum against OmpA protein²⁹ and protein A coupled to Sepharose³⁰. *b*, Strains SD12 (lane 5, wild-type), HD3122 (*pgsA3*) (lane 6, low PG and CL) and SD11 (lane 7, low CL), each containing plasmid pJP29 which carries the *phoE* gene²⁸, were induced for PhoE protein synthesis by growth at 37°C in low phosphate medium³¹, supplemented with chloramphenicol (25 µg ml⁻¹). Whole cells were pulse-labelled with [³⁵S]methionine for 10 s at 30°C. Strain CE1250 was derived from *E. coli* K12 strain CE1224³², containing pHD102, by P1 transduction³³ using strain HD3122 which carries a *Tn10* insertion close to the *pgsA3* allele as donor. A tetracycline-resistant transformant with a low PG content after growth at 43°C was designated CE1250. Curing plasmid pHD102 from this strain followed by plating resulted in pinpoint colonies, indicating that loss of an intact *pgsA3* allele is harmful in this genetic background. All samples were analysed by SDS-polyacrylamide gel electrophoresis³⁴ and the dried gels were subjected to fluorography. The position of some relative molecular mass (*M_r*) standard proteins are shown on the right. For determination of the phospholipid composition, phospholipids were isolated from whole cells or membrane vesicles by solvent extraction³⁵ and separated by thin-layer chromatography on silica gel using chloroform:methanol:acetic acid (65:25:10) as solvent one and chloroform:pyridine:formic acid (60:30:7) as solvent two. The phosphate content in each spot was measured after perchloric acid destruction³⁶.

Phosphatidylglycerol phosphate synthase is a key enzyme in the biosynthesis of phosphatidylglycerol (PG), the major acidic phospholipid in *E. coli*. A mutation in the *pgsA* locus of the *E. coli* genome was described¹⁵, which resulted in undetectable levels of activity of this enzyme and consequently very low levels of PG and cardiolipin (CL), which is derived from PG. Several *E. coli* strains carrying this *pgsA3* allele required the presence of a plasmid (such as pHD102) carrying a functional *pgsA* gene for growth¹⁶, suggesting that these lipids are very important. We used such *pgsA3 E. coli* strains to study the role of the anionic lipids in protein translocation.

Cells of the *pgsA3 E. coli* strain CE1250 were cured of plasmid pHD102 by growth at the restrictive temperature for plasmid replication (43°C). When the PG content dropped to 1–3 mol% of the total phospholipids (analysed as described in Fig. 1), the cells were pulse-labelled with [³⁵S]methionine to follow the biosynthesis of the outer membrane protein OmpA. Immunoprecipitation showed that significantly more precursor (pre-OmpA) was present, compared to the wild-type strain with normal PG levels (Fig. 1*a*, lanes 1 and 3). In both cases the precursor disappeared during a 30-second chase (Fig. 1*a*, lanes 2 and 4). This is the first indication that the rate of translocation of an outer membrane protein across the inner membrane is decreased by reduced levels of anionic phospholipids.

The *E. coli* strain SD12 carrying the *pgsA3* allele is able to grow normally¹⁶ (strain HD3122) although in agreement with previous reports, the mutation caused a reduction in PG and CL from 11 to 1.0 and 3–4 to 0.5 mol% of total phospholipid respectively. This drop in total level of anionic phospholipids

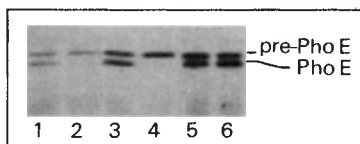


Fig. 2 Effects of low PG or CL content in inner-membrane vesicles on the processing and translocation of *in vitro* synthesized pre-PhoE. Transcription *in vitro* of plasmid pJP29 which carries the *phoE* gene and synthesis of [35 S]methionine labelled pre-PhoE protein were as described previously²⁴. Translation was carried out for 27.5 min at 37°C using a S-135 cell extract of strain SD12 (lanes 1 and 2) or HD3122 (lanes 3–6). SD12 (lanes 1, 3, 5), HD3122 (lanes 2, 4) or SD11 (lane 6) vesicles were added 7.5 min after the translation had been started to a final concentration of 0.5 A_{280} U ml⁻¹, corresponding to 0.2 μ mol phospholipid phosphorus per ml. Protease digestion was carried out by adding 200 μ g ml⁻¹ of proteinase K for 30 min at 22°C and translocated proteins were analysed by fluorography of 12% SDS-polyacrylamide gels. Radioactivity of the protein bands was determined²⁴. Inner-membrane vesicles were prepared²⁴ from whole cells grown in yeast broth at 37°C (lanes 1–4; ref. 27) or 30°C (lanes 5 and 6). In SD11 cells grown at 30°C the CL content is $\sim$ 0.05 mol % of the total phospholipids.

is slightly counterbalanced by an accumulation of phosphatidic acid (1.8%). No significant change in lipid/protein ratio occurred in the inner membrane (0.4 μ mol lipid per mg protein), and nor did the fatty acid composition of the total cellular phospholipids change drastically (7% increase in 18:1c at the expense of 16:1c).

The plasmid pJP29, carrying the *phoE* gene, was introduced into strains SD12 and HD3122 and these were then induced for the synthesis of the major outer membrane pore protein PhoE. Pulse-labelling of the cells showed that more precursor (pre-PhoE) accumulated relative to processed product (PhoE) in the mutant strain HD3122, than in the wild-type strain (Fig. 1b, lanes 5 and 6). Total synthesis of PhoE protein, but not of the cytoplasmic protein chloramphenicol acetyl transferase (CAT), was reduced during the pulse in HD3122. In addition, cells containing the *pgsA3* allele had an elongated shape. Such decreased synthesis of secreted proteins and an elongated shape are often observed in export-defective *E. coli* cells^{17–21}. To discriminate between effects of PG and CL on protein translocation similar experiments were conducted with strain SD11, which is defective in CL synthase²². These cells, when grown at 37°C, have a normal PG but a very low CL content (0.1 mol % of total phospholipid). Compared to the wild-type strain SD12, total PhoE synthesis and translocation were similar (Fig. 1b, lane 7), strongly suggesting that it is the reduced level of PG that is responsible for the effects observed with the *pgsA3*-allele carrying strains.

Cell-free translation-translocation systems²³ allow a more detailed analysis of the influence of low acidic-phospholipid levels on protein translocation. Translocation of PhoE protein across inverted *E. coli* inner-membrane vesicles can be monitored by processing of translocated pre-PhoE by endogenous leader peptidase and by its protection against added protease²⁴.

Pre-PhoE protein was synthesized in such a system supplemented with a cytosolic extract of the wild-type SD12 strain and vesicles of either SD12 or HD3122 (low PG, low CL). Translocation across mutant vesicles was reduced as shown by a decrease in the percentage of total synthesized PhoE which was insensitive to protease digestion because it was sequestered in the vesicles (7.5% and 3.4% for SD12 and HD3122 vesicles, respectively), and by an inhibition of pre-PhoE processing as indicated by the low intensity of the PhoE band (Fig. 2, lanes 1 and 2; processing of translocated pre-PhoE was 56% and 21% for SD12 and HD3122 vesicles, respectively). These effects are not due to the low CL content as there was efficient transloca-

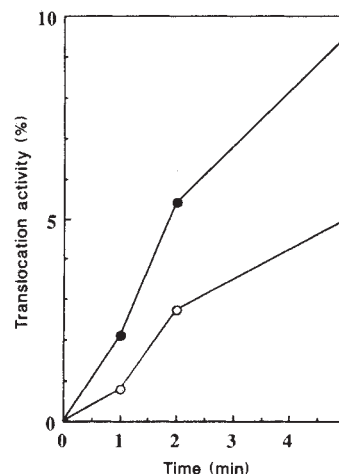


Fig. 3 Kinetics of post-translational translocation of PhoE protein across inner-membrane vesicles of strain SD12 (●) and HD3122 (○). Synthesis of pre-PhoE was carried out for 25 min at 37°C using a cell extract of strain HD3122, after which translation was inhibited by the antibiotic puromycin²⁴. To 40 μ l of the translation system, 10 μ l containing ATP (20 mM), unlabelled methionine (500 μ M) and an optimal amount of inner-membrane vesicles for translocation was added (final concentration 0.5 A_{280} U ml⁻¹) and the incubation was continued at 37°C. At the indicated points of time translocation was stopped by the addition of proteinase K and the translocation activity was determined.

tion and processing of PhoE in SD11 vesicles (Fig. 2, lanes 5 and 6; percentage translocated protein was 30% and 24% for SD11 and control vesicles, respectively). Because the effect of the low PG level on PhoE translocation and processing appears to be stronger *in vitro* than *in vivo* (compare Figs 1 and 2), we considered whether a cytoplasmic factor in the HD3122 lysate might compensate for the decrease in PG content. This appears to be the case, as the mutant lysate produced a threefold stimulation of translocation activity of both SD12 and HD3122 vesicles (Fig. 2, lanes 3 and 4; 20.5% and 8.8% total translocation for SD12 and HD3122 vesicles, respectively). The nature of this factor is not known.

Figure 3 confirms that the mutant HD3122 vesicles are less effective in pre-PhoE translocation than the wild-type strain in a post-translational translocation experiment. Moreover, these data directly show that it is the rate of translocation which is decreased by lowering the PG level. After 5 min translocation levels off, and it even decreases after longer incubation times, presumably due to digestion of the proteins. The efficiency of processing of the translocated pre-PhoE was independent of time. Both SD12 and HD3122 vesicles generated a membrane potential (measured as described in ref. 24) which was sufficient for maximal translocation activity and dissipation of which by 5 μ M CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) reduced translocation to a similar low level. Therefore, we assume that the involvement of PG in protein translocation is not the indirect result of changes in membrane potential.

Although protein processing and translocation need not be strictly coupled processes in *E. coli*²⁵, it could be argued that inhibition of processing causes blocking of export sites, thereby reducing the translocation efficiency of pre-PhoE. Consistent with this suggestion, the activity of purified *E. coli* leader peptidase on pre-PhoE, in a mixed micellar β -octyl glucoside detergent system, was stimulated two to fivefold by PG but not by the zwitterionic phosphatidylethanolamine or phosphatidylcholine. Translocation itself, and not just processing, however, is affected in the HD3122 vesicles as deduced from experiments with a PhoE mutant protein, deleted in the N-terminal mature part from amino-acid position +3 to +18, which is translocated

in vivo across the inner membrane into the periplasmic space without being processed²⁶. This mutant protein is also translocated across wild-type and HD3122 vesicles without being processed. Translocation across mutant vesicles was reduced 40% compared to wild-type vesicles, demonstrating that lack of processing alone is not responsible for inhibition of translocation (data not shown).

Whether PG acts in the protein translocation pathway across the *E. coli* inner membrane in a direct or indirect manner is not yet known. Recent model membrane-experiments¹⁴ with the signal peptide of PhoE and *E. coli* membrane lipids revealed a negatively-charged lipid-specific insertion of the signal peptide into the membrane, resulting in changes in both signal peptide structure and lipid organization. This is in agreement with a role for signal-peptide/lipid interactions for protein translocation across the *E. coli* inner membrane.

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X-ray structure of a DNA hairpin molecule

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We have solved the crystal structure of a synthetic DNA hexadecanucleotide of sequence: C-G-C-G-C-G-T-T-T-C-G-C-G-C-G, at 2.1 Å resolution, and observed that it adopts a monomeric hairpin configuration with a Z-DNA hexamer stem. In the T₄ loop the bases stack with one another and with neighbouring molecules of the crystal, and not with base pairs of their own hexamer stem. Two thymine T10 rings from different molecules stack between the C1-G16 ends of a third and a fourth hairpin helix, in a manner that suggests T-T base 'pairing' and simulates a long, 13-base-pair helix. Although such T-T interactions would not be present in solution, they illustrate a remarkable tendency of thymines for self-association. Purine-purine G-A base pairs are known to exist in the *anti-anti* conformation with an increase in local helix width¹⁻⁴; it may be that more serious consideration should be given to the possible existence of pyrimidine-pyrimidine C-T base pairs with decreased local helix width, particularly where several such base pairs occur sequentially.

The 16-mer structure was solved by molecular replacement, using idealized B-DNA and Z-DNA hexamers to search for the position of the hairpin stem in the crystal. The Z helix gave better agreement with the X-ray data than the B, and was selected for further refinement. As Figs 1 and 2 show, the six-base-pair stem is a normal left-handed Z-DNA helix, like those observed in the crystal structures of C-G-C-G-C-G (ref. 5) or ¹³C-G-¹³C-G-¹³C-G (ref. 6). Glycosyl bond conformations alternate between

anti at Cs and *syn* at Gs. Deoxyribose ring conformations are in the C2'-*endo* family at Cs and the C3'-*endo* family at Gs, except for the 3'-terminal guanine sugars at the two ends of the Z stem. These are C2'-*endo*, as is generally found at this position in Z structures. The adoption by interior guanines of C3'-*endo* sugar puckering appears to be a requirement for a continuing Z-helical backbone chain, rather than any intrinsic preference for *syn* guanines for the C3'-*endo* conformation. Even at G6 in the hairpin hexadecamer, where the chain continues but in the form of a loop rather than a Z helix, the guanine sugar conformation relaxes from C3'-*endo* to C2'-*endo*.

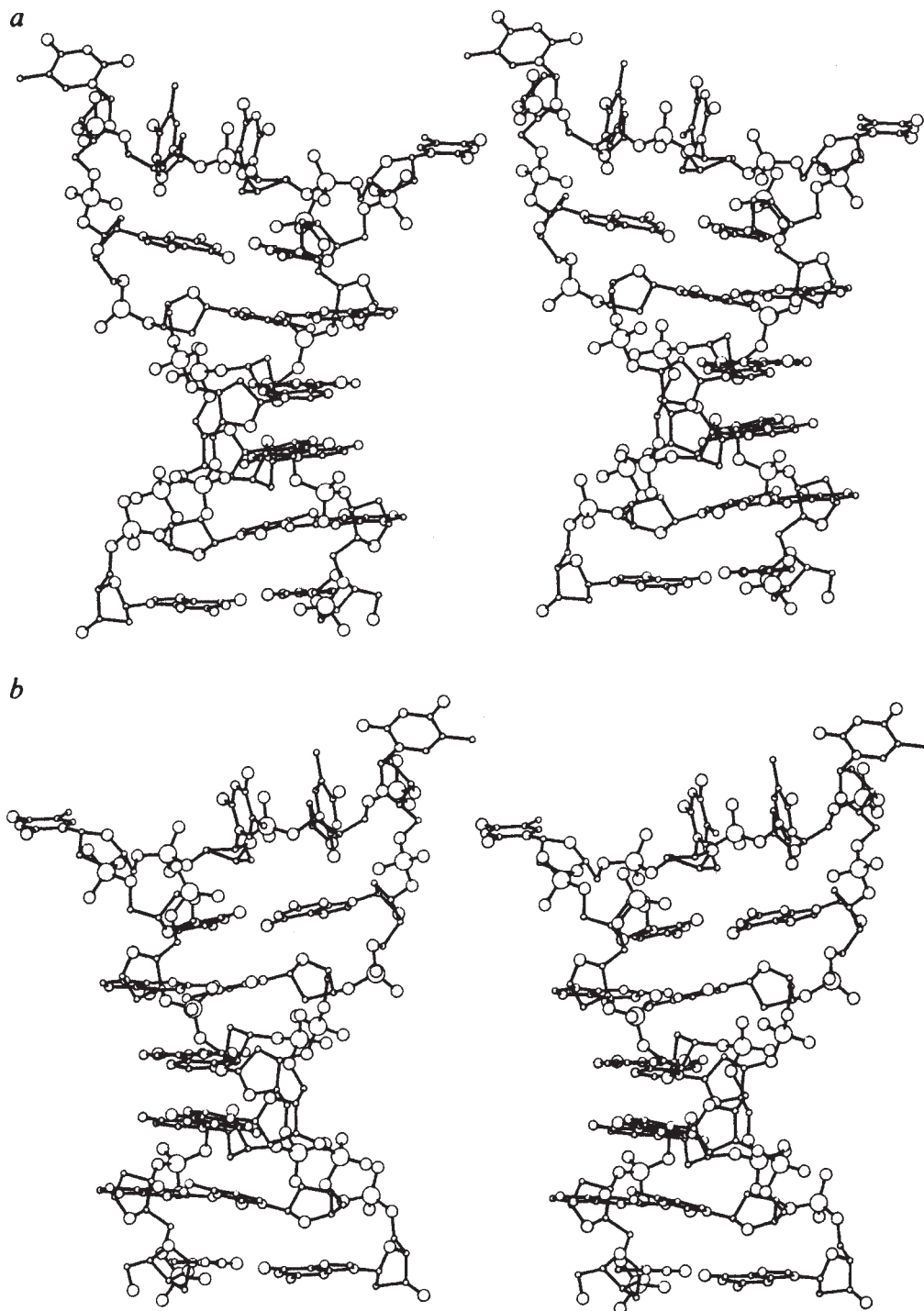
The growth of crystals of DNA hairpins has been intractable in the past. This is probably because monomer hairpins are an intermediate state in a three-state equilibrium bounded on one side by isolated random-coil chains, and on the other by double helices⁷⁻¹⁰. The shift from hairpin monomers to helical duplexes is favoured by lower temperature and by higher DNA and salt concentrations, all of which also are conditions favouring crystal growth. The present C-G-C-G-C-G-T-T-T-C-G-C-G-C-G may be particularly favoured for hairpin crystallization because its sequence permits it to adopt another high-concentration, high-salt alternative to the duplex: a Z helix.

The four-base-pair loop is close to the minimum sterically acceptable length¹¹⁻¹³. The path of the chain was determined by difference-map methods, and given a final check by the difference map shown in Fig. 3a. There is little sign of any tendency for loop bases to stack against the final G-C base pair of the helix. The only element of the loop that stacks against base pair G6-C11 is S9, the sugar ring of thymine 9. In place of such stacking on G6-C11, the loop builds a minstack of its own consisting of sugar S7 and thymine rings T8 and T9 at right angles to the base planes of the stem (Fig. 3b).

Sugar O4' atoms have a tendency to nest against neighbouring aromatic rings. It is a characteristic feature of Z-DNA that each cytosine sugar-oxygen is packed against the guanine ring that follows it in a 5'-3' direction. In the present structure, G6 is sandwiched between the oxygens of sugar S5 in the stem and S8 in the loop. The oxygen of S7 nests against T8, and that of S9 against S8. Only with S10 is the deoxyribose sugar exposed to the surroundings.

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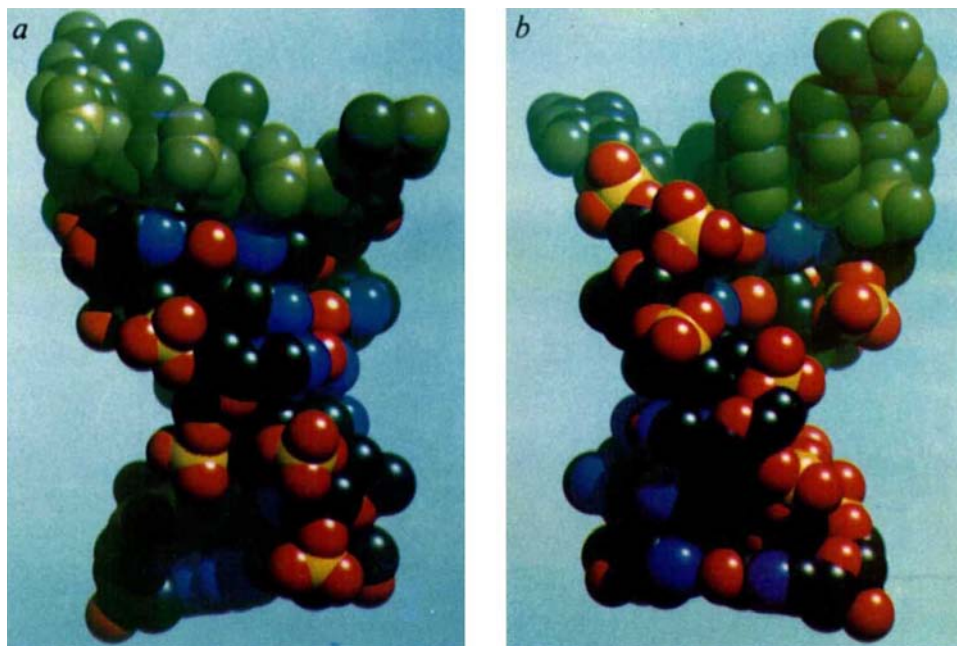
The only previous X-ray crystal structure of a nucleic-acid hairpin loop is that of the three arms of transfer RNA^{3,4,14-16}. But these are poor models for the present T₄ loop. The D and TΨC arms of tRNA are folded into a complex tertiary structure, and the anticodon loop has considerably more freedom, with seven unpaired bases rather than four. Two of the loop bases are stacked on top of the base-paired adenine at the 5' end of the loop, and the other five, including the anticodon triplet, are stacked on top of the paired base as the 3' end of the loop. All of the bases in the loop are roughly parallel to one another and to the base pair that closes off the loop.

A very similar stacked-base arrangement has been proposed for the T₄ loop of C-G-C-G-T-T-T-C-G-C-G, on the basis of nuclear magnetic resonance (NMR) measurements in solution

and distance geometry calculations¹⁷. That hairpin has a B-DNA stem, and loop thymine rings that are generally stacked parallel to the final base pair on top of the stem, as with tRNA. In the nomenclature of our hexadecamer, their thymines T7 and T10 stack on top of the final G-C base pair of the stem, and their T9 stacks more loosely against T10. Only T8 remains unstacked, although roughly parallel to the other bases of the loop and to the B-DNA stem.

The difference in T₄ loop conformation between the X-ray and NMR studies probably reflects differences between crystal and solution environments, and illustrates the flexibility of a four-base loop. Our loop as viewed in Fig. 3a is by no means fully extended, and a different conformation could easily be imagined under different circumstances. The geometry of attach-

Fig. 2 *a*, Space-filling representation of the Z hairpin, viewed from the front as in Fig. 1*a*. Atoms in the hexamer stem are given standard colours: P=yellow, O=red, N=blue, C=black. Atoms in the hairpin loop are in different shades of green for emphasis. In this view the three loop phosphates, P8-P9-P10 from left to right, are especially prominent, as is the extended thymine ring of T10. *b*, The hairpin from the back as in Fig. 1*b*, giving special prominence to the stacked rings of T8 and T9, and to the ring of T7 at upper right.



ment of a T_4 loop to a B-DNA stem versus a Z-DNA stem would not force adoption of one conformation over another. Although the phosphate-phosphate distance across the helix, 3' to a guanine on one strand and 5' to a cytosine on the other, is greater for B-DNA than for Z-DNA, ~ 17.5 Å versus 15.5 Å, respectively, enough torsional freedom exists around backbone bonds to fit the same T_4 loop to both stems. A loop structure such as we see in the crystal will not adequately explain the NMR observations (B. Reid, personal communication). It may be that, with isolated hexadecamer molecules in solution, bases of the T_4 loop favour stacking onto bases of the stem, whereas the proximity of neighbours in the crystal allows stacking energy to be gained intermolecularly.

Thymine T10 in our hexadecamer structure is involved in a curious intermolecular interaction. In most other crystal structures of Z-DNA oligomers, cylindrical double helices stack end to end in a manner that approximates a continuous helix through the helix. With the present hairpin hexadecanucleotide, one might have expected two hairpins to stack with their terminal C1-G16 base pairs in contact, forming a pseudododecamer capped at each end by a loop. What is actually observed is a surprising variant of this: the two C1-G16 base pairs are held apart by the insertion of two T10 thymine rings from different neighbouring molecules, constructing a thirteenth 'base pair', T-T (Fig. 4).

The two thymines in Fig. 4, related by a crystallographic twofold axis, exhibit a strong negative propeller twist of -23.6° . The O2-O2 separation between thymines of 3.1 Å could be a close-packing contact. But the N3-N3 separation of 2.6 Å and the O4-O4 distance of 2.5 Å suggest hydrogen bonding. Such bonding could not occur between thymines in their normal tautomeric forms. But if one base were to adopt the *enol* tautomer, as in Fig. 5, then two hydrogen bonds could be formed corresponding to the observed short inter-thymine distances. Such *keto-enol* pairs probably would be oriented randomly throughout the crystal lattice, and might even be in a state of dynamic equilibrium with respect to hydrogen atom motion^{18,19}. The resolution of the X-ray analysis is insufficient to shed any light on this issue.

A possible alternative to the *keto-enol* pair shown in Fig. 5 might be *keto-keto* wobble pairs, randomly disordered through the crystal. But this would require a left-right elongation of the T10 ring density at the bottom of Fig. 3*a* which is not in evidence.

More extensive difference-map analyses and Konner-Hendrickson refinement of wobble pairings (R.C., K.G. & R.E.D., in preparation) do not support the wobble structure.

Although this pseudo-13-mer helix is a consequence of crystal packing and would not be present in solution, it illustrates the strong influence that base pairing and stacking have upon helix formations. As has been noted before in connection with the origin of positive propeller twist in right-handed DNA²⁰⁻²², base-plane stacking appears to be the dominant factor in helix formation, with the sugar-phosphate backbones serving mainly to provide an added measure of stability in the face of thermal denaturation. There is an inherent predisposition of base pairs toward helical stacking, whether a connecting backbone is present or not.

Values of local helix parameters of the 'helix' depicted in Fig. 4 all lie within acceptable limits observed in other helices. Considering one strand of the 'helix'—bases C15 and G16 of one molecule, T10 of a second, and C1 and G2 of a third—the values of the rise per residue, h , are reasonable: 3.06 Å, 3.76 Å, 3.99 Å and 3.35 Å. The global twist angle, t_g , between one C1-G16 base pair and the T-T pair is 26° . Roll angles, θ_R , between base planes along one strand of the 'helix' of Fig. 4, or along the progression C15-G16-T10-C1-G2, are $+5^\circ$, $+19^\circ$, -12° and $+6^\circ$, and tilt angles θ_T are $+13^\circ$, $+6^\circ$, -1° and $+1^\circ$. (For definitions of local helix parameters, see ref. 20 and the appendix to ref. 21.)

The T-T base pair might at first glance be dismissed only as a crystal packing artefact, irrelevant to DNA structure in solution. Yet recent enthalpy measurements on solution transitions of (C-G)₃-T-T-T-T-(C-G)₃, identical to our sequence except for an additional thymine in the loop, give credence to T-T interactions²³. Their enthalpy of transition from duplex to hairpins is 23 kcal per mole of monomer strand, and that from hairpin to single strand is 58 kcal per mole. To a first approximation the enthalpy of unstacking and unpairing bases in the hairpin to single-strand conversion is $58/6 = 9.7$ kcal per base pair. If we ascribe the enthalpy of transition from one duplex to two hairpins solely to destacking and unpairing of the five T-T pairs at the centre of the duplex, then very nearly the same enthalpy is obtained, that is $2 \times 23/5 = 9.2$ kcal per base pair. The stability of T-T pairs within their duplex is nearly that which would be expected if true T-T base pairs were present, as in Fig. 5.

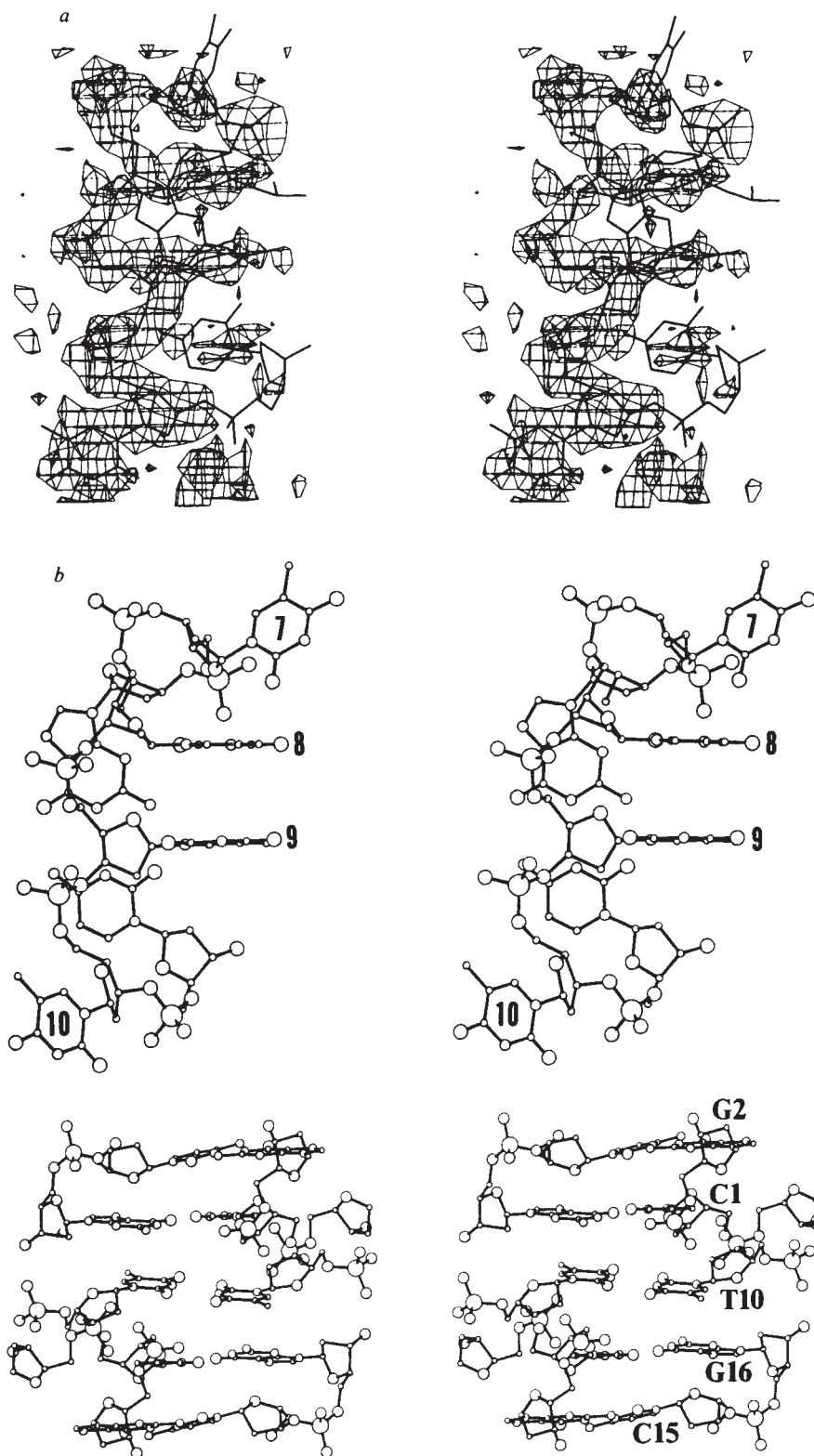


Fig. 3 The T7-T8-T9-T10 loop, viewed from the top looking down the axis of the Z-DNA stem. Base pair G6-C11 is visible underneath the loop. In both views, T7 is at the top, and the chain snakes its way downward to T10 at bottom. *a*, ($F_o - F_c$) difference electron density map, with nucleotides T7 through T10 deleted from the final phasing model to verify that they are restored correctly by the X-ray data (framework contours). *b*, Stereo skeletal view of the final model, in nearly the same orientation. After Hendrickson-Konnert refinement of the Z-DNA stem, the hairpin loop and 70 water molecules were located in several stages of examination of difference maps and refinement of partial structures, as described elsewhere (R.C., K.G. & R.E.D., in preparation). The final R factor is 23.4% for all reflections or 20% for the two-sigma data. The X-ray intensity data and final refined coordinates have been deposited in the Brookhaven Protein Data Bank.

Fig. 4 Stacking of T10 thymine rings (centre) between ends of neighbouring helices (above and below) to build a pseudo-helix. Bases along the right strand of this 'helix' are labelled. They map onto the left strand via rotation about the crystallographic twofold axis perpendicular to the page, through the centre of the diagram. The two T10 rings are sufficiently close to suggest some kind of tautomerization and hydrogen bonding involving N3s and O4s.

In discussion of possible base pair mismatches^{19,24}, purine-pyrimidine mispairs have been regarded as most probable. Purine-purine mispairs generally have been assumed to have one purine in the *syn* glycosyl bond conformation, an arrangement that retains the standard Watson-Crick separation of 10.5 Å between C1' sugar atoms at the connections to the helix backbones. Purine-purine mispairs with both bonds *anti* have been regarded as less probable because they would enlarge the C1'-C1' distance beyond that of a standard DNA helix, to 12.5 Å. Pyrimidine-pyrimidine mispairs have been largely

ignored because their optimal C1'-C1' distance of 8.4 Å could not bridge the gap between chains in a standard helix. But the X-ray structural analysis of C-C-A-A-G-A-T-T-G-G (ref. 1) showed that a B-DNA backbone could expand locally to accommodate the larger dimensions of an *anti-anti* G-A base pair. The converse may also be true: that the helix backbone is sufficiently flexible in principle to incorporate C-T base pairs (Fig. 4) with roughly the geometry of the T-T pairs found in this structure. This might be especially easy if several C-T pairs occurred in succession, allowing the helix backbone to assume

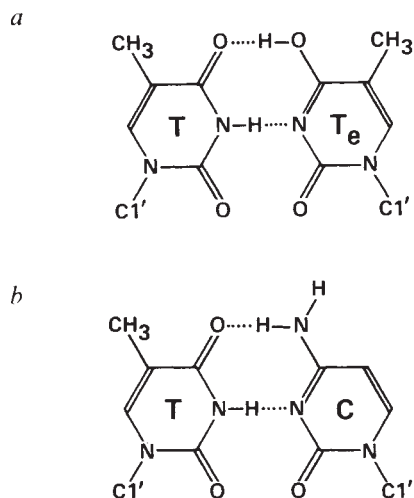


Fig. 5 Pyrimidine-pyrimidine base pairings. *a*, Thymine in its standard tautomer, T, paired with the *enol* tautomer, T_e . *b*, Thymine paired with cytosine, both in their normal tautomeric forms. Actual distances between N and O atoms in the T-T base pair of the hairpin crystal structure are: O4-O4 (top), 2.5 Å; N3-N3 (centre), 2.6 Å; O2-O2 (bottom), 3.1 Å. The C1'-C1' distance in these base pairs should be ~8.4 Å, compared with 10.5 Å for normal Watson-Crick pairs and 12.5 Å for an *anti-anti* G-A purine-purine base pair. The actual C1'-C1' distance between T10 bases observed in this hairpin crystal structure is 8.6 Å.

a narrower local conformation. The self-complementary sequence C-G-C-G-C-T-C-T-C-G-C-G, a variant of the type of sequence C-G-C-x-x-x-x-G-C-G which has proven so tractable ever since the original Drew dodecamer²⁵, might be a particularly suitable candidate for study.

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Gene amplification and expression in plants by a replicating geminivirus vector

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The use of plant DNA viruses as vectors for the transfer of foreign genes to plants offers two potential advantages over other, existing methods of producing transgenic plants. First, these viruses systematically infect whole plants, thus obviating the need for the difficult and time-consuming step of regeneration from transformed single cells or protoplasts. Second, the viruses replicate as separate, autonomous entities within the plant's cells so that any gene cloned in a plant DNA-virus vector would be amplified to high copy number, a feature that differs from methods that produce transgenic plants by the chromosomal integration of foreign DNA. To date, attention has focused on the development of vectors based on the cauliflower mosaic virus (CaMV), but their use is hampered by the narrow range of plants infected by CaMV, and by practical limitations on inserting foreign DNA that are imposed by the biology of CaMV. Here we describe the use of vectors based on the gemini tomato golden mosaic virus (TGMV) to introduce the neomycin phosphotransferase (*neo* gene) into plants. Our results indicate that geminivirus-derived vectors should be useful not only for amplification of gene expression by the systemic infection of plants, but also for heritable gene amplification by the integration of stable master copies of the vector into the plant chromosomal DNA.

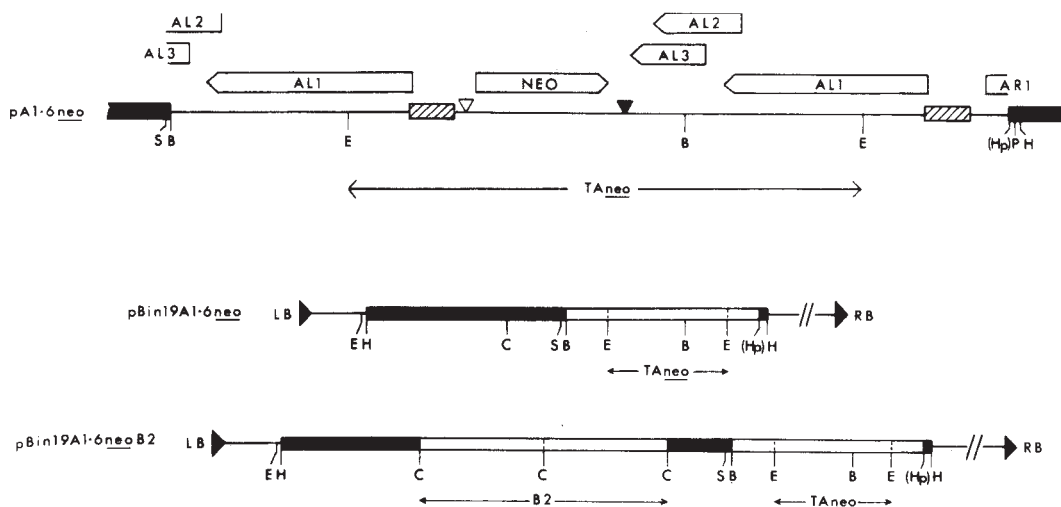
TGMV has a genome composed of two single-stranded DNA components, A and B, each about 2.5 kilobases (kb) in size¹. DNA A encodes the coat protein and functions required for replication in single cells²⁻⁵, whereas DNA B is necessary for cell-to-cell spread of the virus and systemic infection of plants³. Several properties of TGMV suggested that it could provide vectors for amplifying genes to high copy number in plants⁶. (1) Double-stranded DNA clones of the viral DNA are infectious and easily manipulated⁷⁻¹⁰. (2) The virus replicates to high copy number in epidermal, mesophyll and phloem-associated cells¹¹. (3) In transgenic plants containing dimers of DNA A integrated into the chromosome (A2 plants), monomeric replicating DNA A molecules are produced^{13,4,9}. (4) The virus coat protein is not required for infection of plants^{8,9,12}. To test the utility of TGMV as a vector for foreign genes we constructed a chimaeric DNA, *Taneo* (Fig. 1), in which most of the coat protein gene including the initiation codon, has been deleted and replaced with the *neo* gene from *Tn5* which thus lies between the putative coat protein transcriptional control signals (promoter and polyadenylation signals)¹³.

To infect plants with this chimaeric DNA we used *Agrobacterium*-mediated inoculation (agroinfection¹⁴ or agroinoculation¹⁰), a method which has been shown to be applicable to both dicotyledonous and monocotyledonous plants^{14,15} and to be the most efficient way of producing systemic infections of plants with both wild-type TGMV DNA and coat-protein deletion mutants^{9,10}. For this purpose we constructed pA1.6*neo*, a partial dimer of *Taneo* cloned into a pEMBL vector (Fig. 1). pA1.6*neo* contains two copies of open reading frame AL1 which is believed to encode a protein important for DNA replication^{2,8}, and two copies of the 'common region' a sequence of 200 bases which is almost identical in DNAs A and B, and which is thought

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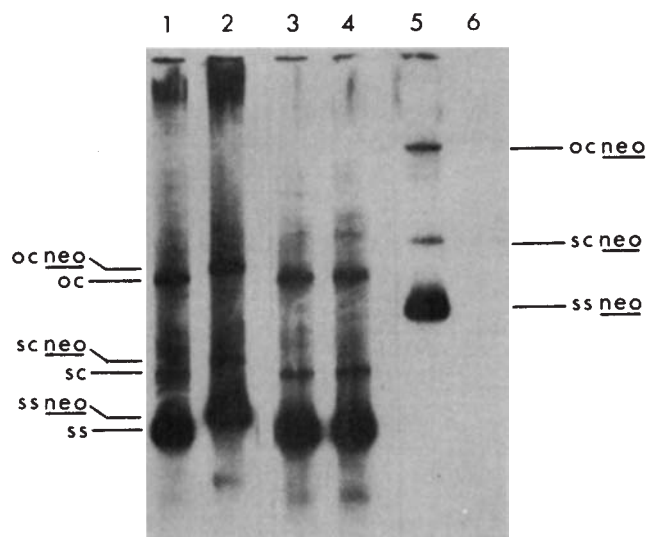
Fig. 1 Structures of pA1.6neo, pBin19A1.6neo and pBin19A1.6neoB2. The open and closed triangles indicate the positions of the putative promoter (TATA) and polyadenylation (AATAAA) sequences^{3,28} upstream and downstream respectively of the TGMV coat protein gene (open reading frame AR1)⁵ which has been replaced by the *neo* gene from *Tn5*. Thick black lines represent pEMBL9 sequences, hatched boxes represent the 200 bp common region of TGMV DNA A and open boxes with arrows represent open reading frames in TGMV DNA A (AL1, AL2, AL3, AR1) or *Tn5* (*NEO*)^{13,22}. B =



*Bam*HI, *C* = *Cla*I, *E* = *Eco*RI, *H* = *Hind*III, *P* = *Pst*I, *S* = *Sma*I. (Hp) = *Hpa*I site of DNA A destroyed in the construction of pA0.6 (see below). The positions of *T*Aneo between the two *Eco*RI sites, and the head-to-tail dimer of DNA B (B2) between the two *Cla*I sites, and the head-to-tail dimer of DNA B (B2) between the two *Cla*I sites, are indicated. LB and RB represent the left and right T-DNA border sequences of pBin19 (sequences outside the T-DNA borders are not shown).

Methods. Plasmid pTAneo2 (ref. 13) consists of *T*Aneo cloned into the *Eco*RI site of pAT153. Plasmid pA0.6 (ref. 9) consists of the 1,664 bp *Bam*HI–*Hpa*I fragment of DNA A cloned between the *Bam*HI and *Hinc*II sites of a pEMBL9 vector which had been modified by destroying the *Eco*RI site in the polylinker region. Plasmid pB2 (ref. 26) consists of a head-to-tail dimer of DNA B cloned into the *Cla*I site of pEMBL9. *T*Aneo was excised from pTAneo2 with *Eco*RI, separated from the vector by agarose gel electrophoresis²⁵, purified using GeneClean according to the manufacturer's (Stratagene) instructions and then ligated into the *Eco*RI site of pA0.6. A recombinant with the full-length and partial-length DNA A moieties joined together in the same orientation (pA1.6neo) was identified by restriction analysis. Plasmids pA1.6neo and pB2 were digested with *Bgl*II for which there are two sites in the pEMBL moiety⁹ but no site in DNA A, DNA B or *neo*. The fragments containing the partial A dimer and the B dimer respectively were isolated by gel electrophoresis and ligated together to produce pA1.6neoB2. Plasmids pA1.6neo and pA1.6neoB2 were digested with *Hind*III, for which there is one site in the pEMBL9 moiety but no site in DNA A, DNA B or *neo*, and ligated into the *Hind*III site of pBin19 (ref. 16) to give pBin19A1.6neo and pBin19A1.6neoB2 respectively.

Fig. 2 Electrophoretic analysis of DNA forms produced during agroinfection of untransformed *N. tabacum* plants. Lanes 1, 3, and 6, 5 µg of DNA from plants agroinfected using pBin19A2B2; lanes 2, 4, and 5, 5 µg of DNA from plants agroinfected using pBin19A1.6neoB2. Southern blots were hybridized with [³²P]-labelled A-specific (lanes 1 and 2), B-specific (lanes 3 and 4) or *neo*-specific (lanes 5 and 6) probes. oc, sc and ss = open circular, supercoiled and single-stranded DNA forms respectively of DNA A or DNA B. ocneo, scneo and ssneo = open circular, supercoiled and single-stranded DNA forms respectively of *T*Aneo. Designations of bands on the left refer to lanes 1 to 4, while those on the right refer to lanes 5 and 6. Electrophoresis of DNA in lanes 5 and 6 was for a shorter time than that of DNA in lanes 1 to 4.



to contain an origin of DNA replication². The duplications are necessary to ensure formation of monomeric replicating DNA molecules in the plant cell, presumably either by intramolecular recombination or by a replicative mechanism involving the two origins of replication^{9,10}.

pA1.6neo and pA1.6neoB2, a derivative of pA1.6neo with an additional head-to-tail dimer of DNA B, were cloned between the T-DNA border sequences of the Ti plasmid vector pBin19 (ref. 16) to give pBin19A1.6neo and pBin19A1.6neoB2 respectively (Fig. 1). These plasmids were then introduced⁹ into *A. tumefaciens* strain PC2669, which contains plasmid pTiC58 to

supply the virulence functions necessary for DNA transfer from bacterium to plant.

When *Nicotiana tabacum* cv. Samsun plants were inoculated by stem injection⁹ with *A. tumefaciens* containing pTiC58 and pBin19A1.6neoB2, 4 out of 20 plants were found to be infected when assayed after 21 days by dot hybridization of leaf DNA with ³²P-labelled A and B component-specific TGMV probes. Agroinoculation of transgenic B2 plants (plants containing a head-to-tail dimer of DNA B integrated into chromosomal DNA) was more efficient; 18 out of 20 plants were found to be infected after 21 days. In comparison, when 20 plants were

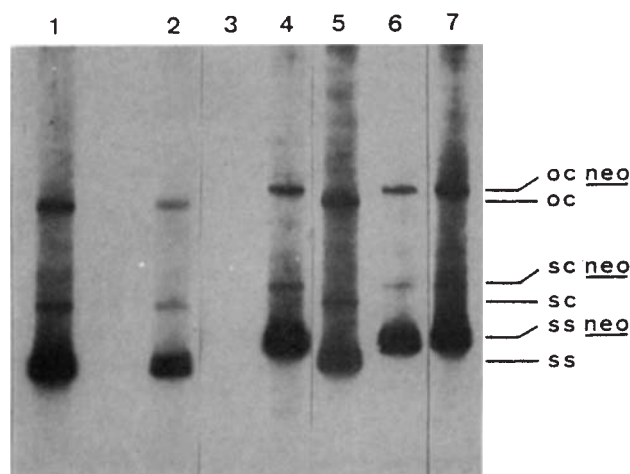


Fig. 3 Electrophoretic analysis of DNA forms in untransformed and transgenic *N. tabacum* plants. Each lane contains 5 µg of plant DNA. Lane 1, DNA from an untransformed plant agroinfected using pBin19A2B2; lane 2, DNA from a transgenic A2 plant; lane 3, DNA from a transgenic TAneo plant; lane 4, DNA from a transgenic A1.6neo plant; lane 5, DNA from a transgenic B2 plant agroinfected using pBin19A2; lane 6, DNA from an untransformed plant agroinfected using pBin19A1.6neoB2; lane 7, DNA from a transgenic B2 plant agroinfected using pBin19A1.6neo. The Southern blot was hybridized with a ³²P-labelled probe specific to DNA A. With the length of exposure used in this blot, the chromosomal copies of DNA A (lane 2) or TAneo (lanes 3 and 4) were not detected, indicating that the number of chromosomal copies was small compared to the number of replicating copies. A lower exposure was used for lanes 5 to 7. Bands are labelled as in Fig. 2. The doublet of single-stranded DNA seen in some lanes is due to separation of the circular and linear forms. Methods are as in Fig. 2 and Table 1.

agroinoculated with pBin19A2B2, which contained head-to-tail dimers of DNAs A and B inserted between the T-DNA borders of pBin19 (ref. 9), 19 became infected and developed a mild chlorosis around the leaf veins after 5–10 days.

Gel electrophoresis and Southern blotting of DNA from plants infected with pBin19A2B2 revealed TGMV single-stranded DNA and supercoiled and open-circular double-stranded A and B DNA forms of unit-length (Fig. 2, lanes 1 and 3), as found previously⁹. Similar single and double-stranded DNA forms were detected in plants infected with pBin19A1.6neoB2 (Fig. 2, lanes 2 and 4) or pBin19A1.6neo (Fig. 3, lane 7), except that those detected by the A-specific probe were of lower mobility, reflecting the larger size of TAneo compared to DNAs A and B. A neo-specific probe detected single and double-stranded forms of TAneo in plants infected using pBin19A1.6neoB2, (Fig. 2, lane 5), but did not hybridize to DNA from plants infected using pBin19A2B2 (Fig. 2, lane 6). It is clear that TAneo is formed from A1.6neo, and replicates and spreads systemically through the plant together with DNA B, in an analogous way to DNA A and DNA B.

It has been shown that the ability of monomeric DNA molecules to form and replicate in transgenic A2 plants is inherited as a mendelian trait³. To determine if a heritable amplification system could be developed by the integration of master copies of the vector into the plant chromosomal DNA, transgenic plants containing A1.6neo integrated into their chromosomal DNA were produced. As in plants infected by agroinoculation with pBin19A1.6neoB2 (Fig. 3 lane 6) or pBin19A1.6neo (Fig. 3, lane 7), Southern hybridization revealed bands corresponding to the single-stranded and double-stranded supercoiled and open circular forms of TAneo DNA (Fig. 3, lane 4). These bands again migrated more slowly than the corresponding DNA A forms (Fig. 3, lanes 1, 2, 5). No free

Table 1 Gene copy numbers and neomycin phosphotransferase (NPT) activity

Plant	Average gene copy number per amphidiploid genome*	Relative NPT activity†
1. Transgenic pBin19	7	1
2. Transgenic TAneo	7	3.5
3. Transgenic A1.6neo	75	40
4. Untransformed agroinfected using pBin19A1.6neoB2	160	80
5. Transgenic B2, agroinfected using pBin19A1.6neo	490	240

Plants transgenic for pBin19, TAneo or A1.6Neo were produced as follows. pTAneo3, a clone of TAneo in the EcoRI site of pEMBL9, was cut with HindIII and cloned into the HindIII site of pBin19 (ref. 16) to form pBin19TAneo. Plasmids pBin19, pBin19TAneo and pBin19A1.6neo were mobilized into *A. tumefaciens* LBA4404 (pAL4404) and plants were transformed as described previously⁹. For determination of TAneo copy numbers, 5 µg aliquots of DNA isolated²⁵ from untransformed, healthy *N. tabacum* was mixed with known amounts of pTAneo3, and 5 µg aliquots of DNA from each of plants 2–5, were digested with EcoRI, electrophoresed in an agarose gel²⁶, Southern blotted and hybridized with a ³²P-labelled, A-component-specific probe²⁶. The band corresponding to linear TAneo double-stranded DNA was located by autoradiography, cut out and scintillation counted. The pBin19 copy number of plant 1 was determined in a similar way, except that the DNA was digested with PstI and hybridization was with ³²P-labelled pBin19. The amphidiploid genome weight of *N. tabacum* was taken as 7.8 pg²⁷. The TAneo copy number in plant 3 includes chromosomal copies plus monomeric replicating copies; however, the chromosomal copy number is small compared to the replicating copies (see Fig. 3). NPT activity in plants 4 and 5 arises from chromosomal copies of TAneo and/or the neo gene of pBin19, as well as the monomeric replicating copies of TAneo. The chromosomal copy number of a range of plants transformed using pBin19 and derivatives was in the range 1–7 and the contribution to NPT activity from expression of the chromosomal neo genes is likely to be very small (as in plants 1 and 2).

* Refers to pBin19 for plant 1 and double-stranded DNA forms of TAneo for plants 2–5.

† Determined as described by McDonnell *et al.*²⁴

TAneo DNA was detected in transgenic plants containing only a monomeric copy of TAneo (Fig. 3, lane 3).

A functional neomycin phosphotransferase enzyme was formed in plants containing replicating TAneo DNA and enzyme activity correlated well with the average copy number of the double-stranded DNA forms of TAneo (Table 1).

The only other DNA plant virus vector to be described is based on CaMV¹⁸. Geminivirus vectors should have several advantages over caulimovirus vectors. (1) As a group geminiviruses infect a wide range of dicotyledonous and monocotyledonous plants including tobacco, tomato, bean, soya bean, sugar beet, cassava, cotton, maize, oats and wheat¹⁹. In contrast the caulimoviruses are limited to a few families of dicotyledonous plants²⁰. (2) In the TGMV vector a convenient restriction fragment containing the neo gene together with some flanking sequences was directly cloned between the coat-protein promoter and polyadenylation sequences. CaMV genes I, II and III are translated from a polycistronic mRNA by a 'relay-race' mechanism²¹. DNA manipulations using CaMV gene II replacement vectors are laborious because, to maintain this mechanism, flanking sequences have to be removed from genes before insertion so that only a few base pairs (bp) separate the initiation codon of one gene from the stop codon of the preceding gene. (3) The relay-race mechanism also precludes the introduction of different plant promoters into CaMV vectors and hence their use to study control of plant gene expression. (4) The size of

recombinant CaMV DNA vectors is limited by the requirement to package the DNA into virus particles¹⁸. Further investigations will be required to determine the maximum size of DNA that can be inserted into TGMV vectors, but, as the coat protein is deleted and the vector replicates as a plasmid, the insert size will not be limited by packaging constraints. (5) Replication of caulimoviruses, which occurs by reverse transcription²³, is likely to have a higher error rate than that of geminiviruses. (6) Coat protein deletion mutants of TGMV show attenuated symptoms^{9,12} and plants in which the vector replicated were symptomless. Attenuation of symptoms has not been reported for CaMV vectors.

The results show that a bacterial gene can be amplified and expressed in plants by a replicating TGMV vector. Replacement of the *neo* gene in pA1.6*neo* by a polylinker cloning site should allow different genes to be cloned into the vector and expressed under the control of TGMV coat protein transcriptional signals. Such a vector should be useful not only for amplification of gene expression by systemic infection of plants, but also for heritable gene amplification by integration of stable master copies of the vector into the plant chromosomal DNA.

There are many potential uses of such amplifying vectors in plant biotechnology and molecular biology. Production of genetically engineered plants with increased resistance to herbicides, insects or viruses and increased yields of important plant products are applications of potential commercial value. The vectors could also be used for studies of plant gene function either by increasing the efficacy of suppression of gene expression by amplification of antisense RNA¹⁷ or by obtaining high levels of expression of inducible genes. Finally it should be possible to replace the virus coat protein promoter by any plant promoter, thereby extending the use of the vectors to provide rapid assays for factors controlling plant gene expression.

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